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Daily Variations in Protein Synthesis and Tissue Responsiveness to Growth Hormone, Prolactin and Insulin-Like Growth Factor-I in the Gulf Killifish, Fundulus Grandis Baird and Girard.

Zeleke Negatu
Louisiana State University and Agricultural & Mechanical College

DAILY VARIATIONS IN PROTEIN SYNTHESIS AND TISSUE RESPONSIVENESS TO GROWTH HORMONE, PROLACTIN AND INSULIN-LIKE GROWTH FACTOR-I IN THE GULF KILLIFISH, *FUNDULUS GRANDIS* BAIRD AND GIRARD

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by
Zeleke Negatu
B.S., Addis Ababa University, 1977
M.S., Addis Ababa University, 1983
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ABSTRACT

Circadian variations of ^{14}C-glycine incorporation into protein and the responsiveness of muscle tissue to growth hormone, prolactin and insulin-like growth factor-I were examined in gulf killifish, *Fundulus grandis*. Incorporation of label during a 5 h period following ^{14}C-glycine injection was tested at different times of day (LD 12:12). Incorporation varied during the day in all the tissues examined (scale, muscle, liver and intestine). The times of peak incorporations differed among the tissues. Scales showed peak incorporation during the dark hours. Peak incorporation by muscle occurred at the onset of light. These patterns were similar in male and female fish. The daily pattern of incorporation by liver and intestine differed between the two sexes. Growth hormone and prolactin injections enhanced incorporation of the label in muscle but the responses varied as a function of the time of day of injections. Both hormones were most effective at light onset and least effective at light offset. In tissue culture study, recombinant human insulin-like growth factor-I (rhIGF-I) stimulated ^{14}C-glycine incorporation by muscle in a dose-dependent manner. The minimal effective dose was 0.1 ng/ml of incubation medium. Maximal stimulation was obtained with 1 ng/ml. In a time-course study using 1 ng/ml of rhIGF-I, the greatest stimulation was observed 6 h after the onset of a 3 h

exposure to rhIGF-I. Responsiveness of muscle to a 3 h exposure to rhIGF-I examined at 3 different times of a day was greatest at light onset and lowest at light offset. Binding studies using partially purified membrane preparations of muscle demonstrate daily variations in the binding of ^{125}I-rhIGF-I. Greatest binding was observed during the dark period. Daily variations in dissociation constant (Kd) and maximal binding (Bmax) were found at three times of day tested. These studies provide additional evidence that tissue-responsiveness to stimuli vary during a day. Thus, circadian rhythms of tissue-responsiveness coupled with rhythms of growth promoting hormones may produce a temporal interaction between stimuli and responses and play an important role in the regulation of protein synthesis and growth in fish.

INTRODUCTION

The most important physiological role of growth hormone is to promote whole body growth. Its effect on target tissues, however, appears to be more indirect than direct. *In vivo* and *in vitro* studies clearly demonstrate that the action of growth hormone on target tissues is mainly mediated by insulin-like growth factor-I (Daughaday, *et al.*, 1985; Humbel, 1990).

Insulin-like growth factor-I (IGF-I), also known as somatomedin C, is a 70 amino acid single-chain polypeptide that shows considerable homology to insulin and the related peptide insulin-like growth factor II (Zapf and Froesch, 1986). In the circulation, it is bound to specific IGF-binding proteins, which are assumed to play the role of prolonging the half-life of the peptide and regulate the free-peptide available to tissues (Ooi and Herington, 1988). The biologically relevant effects of IGF-I are stimulation of protein synthesis, cell proliferation and cell differentiation (Phillips, *et al.*, 1990). Extensive reviews on various aspects of IGF-I are available in Ooi and Herington (1988), Humbel (1990), Phillips *et al.* (1990), and Cullen, *et al.* (1991).

Studies in mammals show that Insulin-like growth factor-I is primarily produced in the liver and released in the circulation. But IGF-I has been extracted from several

tissues (D'Ercole, *et al.*, 1984) and mRNA for the peptide has been identified in several tissues (Mathews, *et al.*, 1986). Although several organs/tissues are known to produce mRNA for the peptide it is widely accepted that the major source of this hormone is the liver. As such the liver is the major target organ of growth hormone in the stimulation of IGF-I synthesis and secretion. Receptor studies show a wide distribution of growth hormone receptors indicating that growth hormone also acts on tissues other than the liver. This also has been demonstrated in several species of fish, such as salmon (Gray, *et al.*, 1990), eel (Hirano 1991) and trout (Yao *et al.*, 1991). The direct effect of growth hormone on tissues may be mediated through the local production of IGF-I, since growth hormone stimulates synthesis of mRNA for IGF-I in several tissues (Mathews, *et al.*, 1986).

The secretion of IGF-I is regulated directly by growth hormone at least in the liver. In the gulf killifish, Emata (1990) has shown that both growth hormone and prolactin injections increase plasma immunoreactive somatomedin-C reactivity. The stimulatory effect of growth hormone and prolactin on IGF-I secretion, however, is time-of-day dependent. Growth hormone and prolactin injected at different times of day have variable effects on the circulating level of immunoreactive IGF-I, indicating daily variations in the responsiveness of target tissue to these

hormones. Measurements during a 24-h period indicate daily variations in plasma levels of immunoreactive somatomedin-C (Emata 1990). Thus, the daily rhythms of growth hormone and prolactin (e.g. Leatherland, *et al.* 1974; Spieler, 1979; Bates, *et al.*, 1989) coupled with the rhythms of IGF-I response to growth hormone and prolactin may have important roles in the regulation of protein metabolism and growth.

Many behavioral, biochemical and physiological processes of organisms exhibit daily fluctuations which are driven by an intrinsic daily or circadian clock. These daily fluctuations are referred to as circadian rhythms. Studies conducted in the gulf killifish and other vertebrates indicate that circadian rhythms have important roles in the regulation of metabolism. Circadian rhythms, which are a property of all eucaryotic cells (Takahashi, 1991), underly the overt physiological (e.g. hormone level) and behavioral (e.g. spawning, migration) rhythmic processes. An important aspect of circadian rhythm is that the phase relationships between daily rhythms change relative to each other during a year. This conclusion is based, in part, on several studies in the gulf killifish (for review Meier, 1984, 1992). The alterations in phase relations account for changes in the physiological and behavioral conditions of an organism as illustrated in birds (Miller and Meier, 1983a,b), gulf killifish (Emata,

et al., 1985) and hamster (Wilson and Meier, 1989). The physiological conditions of an organism are believed to be the net consequences of temporal synergisms of circadian neural and humoral rhythms coupled to rhythms of tissue responsiveness.

It is well established that vertebrates display daily variations in the circulating levels of hormones. It is apparent that most attention has been given to the rhythms of stimuli (e.g. hormones) and their expressions (physiological, behavioral), whereas rhythms of tissue response to stimuli appear to be neglected. The reason for this may be that it is generally assumed tissue-responsiveness to stimuli is invariable during a day. But there are ample evidences to the contrary. Several studies conducted in the gulf killifish have led to the conclusion that stimuli will produce the greatest response when the daily stimulus peak coincide with the daily peak of tissue-responsiveness, whereas other temporal relations of stimulus and tissue responsiveness rhythms will produce a gradation of lesser effects (for reviews see Meier, 1984 and 1992).

The studies described here were performed with 3 main goals in mind. First, to explore if there is a daily variation in protein synthesis in the gulf killifish, determined from incorporation of labeled glycine. Several aspects of physiological rhythms of the gulf killifish

(reviewed by Meier, 1984, 1992) and more recently daily variation of immune activity (Nevid, 1993) have been extensively studied. However, the roles of circadian rhythms in protein metabolism have not yet been explored.

Although the presence of IGF-I in the plasma of gulf killifish (Emata, 1990) and other fish species (eg. Lindahl, *et al.*, 1985; Funkenstein, 1989) has been reported, its physiologic effect has received little attention. The second goal of these studies was to examine the effect of IGF-I on glycine incorporation into protein and to determine whether its effect, if any, is time-of-day dependent.

The third goal was to see if properties of the receptors for IGF-I undergo daily variations. Variations in tissue sensitivity may possibly be related to variations at the receptor level or to post-receptor events. At the receptor level, variations in responsiveness may result from changes in the number of receptors, which may be a result of the synthesis, degradation or availability of the receptor. It may also be a consequence of changes in receptor affinity for the ligand, which can be modulated by the nature of the membrane. Daily variations in receptor number and affinity to ligand have been reported for several hormones including, for example, insulin (Cincotta and Meier, 1985), serotonin (Hulihan-Giblin, *et al.*, 1993), epidermal growth factor (Scheving, *et al.*, 1989), and the

intracellular receptors for steroid hormones (Francavilla, *et al.*, 1986).

CHAPTER 1

Daily Variation of Protein Synthesis in Several Tissues of the Gulf Killifish, *Fundulus grandis* Baird and Girard*

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Introduction

Studies by our laboratory have led to the conclusion that circadian rhythms have central roles in neuroendocrine regulation of metabolism. Most of these studies relate to the regulation of lipid synthesis and body fat stores in vertebrate species, including teleosts (Meier, 1984; 1992). This conclusion is based on findings that the phases of the rhythms of lipogenic hormones and of target tissue responses to the hormones differ in lean and fat animals so that the temporal interaction of the stimulus and response rhythms regulates the net synthesis of fat (Meier, 1977). Lipogenesis itself is largely restricted to a discreet daily interval as has been shown in *Fundulus grandis* (Horseman and Meier, 1979a) and the Syrian hamster (Cincotta and Meier, 1984 and 1989).

Circadian rhythms may also be important in directing protein metabolism in fish. Growth hormone (GH: Leatherland *et al.*, 1974; Bates *et al.*, 1989), prolactin (PRL: Leatherland and McKeown, 1973; Spieler *et al.*, 1976; Spieler *et al.*, 1979), somatomedin-C (IGF-I, SmC: Emata, 1990), and thyroid hormones (T_4 and T_3: Spieler and Noeske, 1979; Laidley and Leatherland, 1988) have been shown to undergo circadian fluctuations in the plasma of teleost fishes. All of these hormones have been shown to stimulate organismal growth and/or protein synthesis in fish (GH:Inui

and Ishioka, 1985; Foster *et al.*, 1991; PRL: Prack *et al.*, 1980; T_4 and T_3: Narayansingh and Eales, 1975; Matty *et al.*, 1982; Sm-C: Emata, 1990). Protein synthesis itself has been shown to vary during the day in fish scales (Ottaway, 1978) and otoliths (Mugiya *et al.*, 1981). Inasmuch as anabolic hormones may stimulate protein synthesis at different times of day in separate tissues, it seemed appropriate as an initial step in understanding temporal organization in the regulation of growth and development to examine and compare daily rhythms of protein synthesis in several tissues of the gulf killifish, *Fundulus grandis*. Responsiveness of muscle to growth hormone and prolactin was also examined at three different times of a day.

Materials and Methods

Experimental animals. Mature gulf killifish, *Fundulus grandis*, were obtained during winter from a commercial dealer who collected them from brackish water along the Louisiana coast of the Gulf of Mexico. The fish were held on 12-hour daily photoperiods (LD 12:12, light onset at 08:00) for six months prior to experimentation. The water was maintained at a temperature of 24°C and the salinity (Instant Ocean) was held at $5°/_{oo}$. The fish were fed commercial fish food (Tetramin) two to three times a day at variable times, except on the days of the experiments. The

time of feeding was varied because daily stimuli such as feeding and disturbance at fixed times of day are known to alter or influence circadian rhythms of physiological processes (Meier *et al.*, 1973; Spieler, *et al.*, 1977; Weld and Meier, 1984; Boujard and Leatherland, 1992; Mattern, *et al.*, 1993).

Experiment 1. Male fish with a mean body weight of 10.8 ±2.2 (S.E.M.) g were used for this *in vivo* experiment. At six-hour intervals during a day, beginning at light onset, four groups of six fish each were anesthetized in 0.02% tricaine methanesulfonate salt (MS-222, Sigma). Each fish was injected intraperitoneal with 20 µl of ^{14}C-glycine (ICN Biochemicals, specific activity of 51 mCi/mmol), which gave a dose of approximately 2 µCi/fish. The fish were sacrificed with an overdose of MS-222 five hours after the glycine injection, and the scales, muscle, liver and intestine were collected. Scales were taken from the lateral sides of the body and muscle tissue was taken from the caudal peduncle area below the dorsal fin. The scales were dried in an oven (50-60°C) to constant weight, and assayed for radioactivity. The other tissues were frozen for later determinations of protein content and radioactivity.

Experiment 2. ^{14}C-glycine incorporation by tissues of male fish (10.6 ±1.5 g) was also examined *in vitro*. The fish were sacrificed with overdoses of MS-222 at three

different times of a day (5 fish/time) at six-hour intervals beginning at light onset. Scales, muscle, liver and intestine were taken as described in Experiment 1 except that the tissue samples were pooled for each time of day. Muscle, liver and intestine were diced into small pieces of about 2 mm^3, pooled as individual tissue samples, rinsed with fish saline and incubated in Falcon petri dishes containing 5 ml of fish saline with 0.4 μCi/ml of ^{14}C-glycine (Goolish and Adleman, 1983). The composition of the fish saline was 140 mM NaCl, 2.68 mM KCl, 1.51 mM $CaCl_2$, 15 mM $NaHCO_3$, 25 mM HEPES, adjusted to pH 7.4. After four hours of incubation at room temperature (23 $\pm$1°C) the tissues were removed and washed with three changes of saline. The scales were immediately placed in an oven to dry, and the other tissues were frozen until protein and radioactivity determinations were made.

Experiment 3. Female fish with a mean body weight of 9.9 $\pm$1.6 g were used. The protocol followed was the same as in Experiment 1 except that seven fish were used at each of the four different times during a day.

Experiment 4. A total of 189 male fish (mean weight of 2.35 $\pm$ 0.03 g) were used for this experiment. Saline (0.75%) or hormone (20 μg/fish) was administered intraperitoneally in 20 μl volume at 0800, 1400, or 2000 h. At each injection time three groups of fish were injected with either saline (21 fish), ovine growth hormone (21

fish) or ovine prolactin (21 fish) (GH and PRL are gifts from NIDDK). After 1, 3, and 6 h of saline or hormone administration seven fish from each group were injected with ^{14}C-glycine (1 μCi/fish in a volume of 20 μl). The fish were then sacrificed 30 min after the administration of the label, and muscle was removed for the determinations of protein and radioactivity.

Protein and Radioactivity determinations. Tissues, except scales, were homogenized in 2.5 ml Tris buffer, pH 7.4. The homogenates were centrifuged for 15 min at 3000 xg and the pellet discarded. The supernatant was mixed with 1.5 ml of 10% TCA, left for one hour on ice, and centrifuged at 4300 xg for 30 min. The pellet was air-dried and resuspended in 0.2 ml 1N NaOH. 25 μl samples were used for protein determination by the method of Lowry, et al. (1951) using bovine serum albumin (Sigma) as a standard. 50 μl samples were used for the determination of radioactivity using a liquid scintillation spectrophotometer (Beckman 8000). Quenching was corrected by sample channels ratio. ^{14}C-glycine incorporation is expressed as dpm/mg dry scale weight for scales, or as dpm/mg total protein for the other tissues.

Data analyses. Data were analyzed by one-way analysis of variance (ANOVA). Where differences were significant ($p<0.05$) the means were compared using Duncan's multiple

range test (DMRT). All data are presented as the mean ± standard error of the mean (S.E.M.).

Results

The results of the *in vivo* glycine incorporation by different tissues in male fish (Experiment 1) are presented in Figure 1. Glycine incorporation varied during a 24-hour period in each of the tissues (ANOVA p values: scale, $p<0.05$; muscle, $p<0.001$; liver, $p<0.0001$; intestine, $p<0.01$); however, the times of the daily peaks and troughs differed among the tissues. Greatest incorporations occurred during the dark period in scales, during the early photophase in muscle and liver and during late dark and early light in the intestine.

The results of the *in vitro* study in male fish (Experiment 2, Figure 2) virtually duplicated the pattern of incorporation observed in the *in vivo* study of male fish (Experiment 1). As in the *in vivo* study, peak incorporations occurred during the early photophase in muscle, liver and intestine, and lowest values were found in the second half of the photophase. Incorporation by scales differed from the *in vivo* study in that two peaks rather than one, were observed; one during the early photophase and a second during early scotophase.

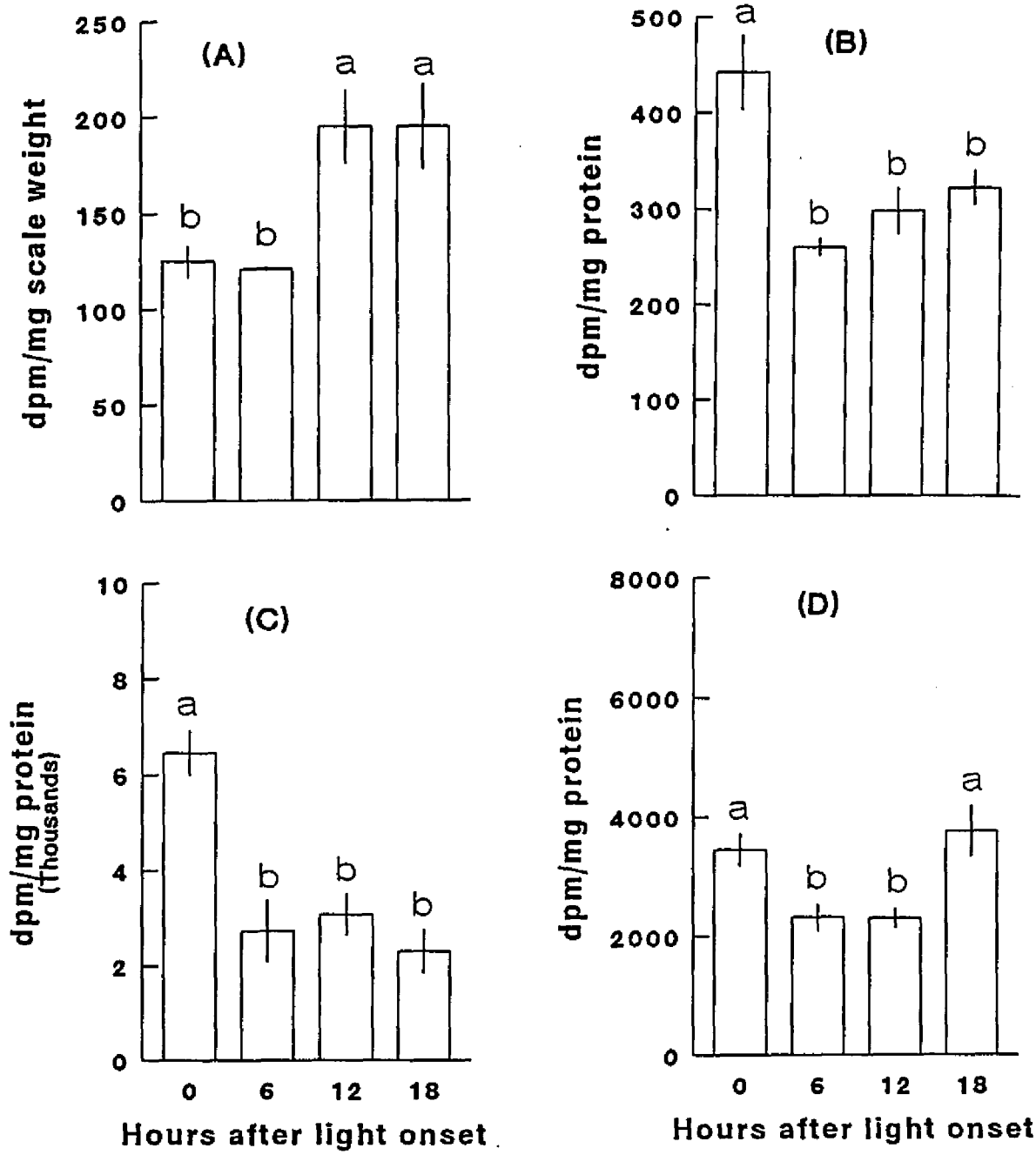


Figure 1. *In vivo* ^{14}C-glycine incorporation by (A) scales (B) muscle (C) liver and (D) intestine of male fish injected with ^{14}C-glycine at four different times during a day. Means with the same small case letter are not significantly different from each other ($p > 0.05$, DMRT)

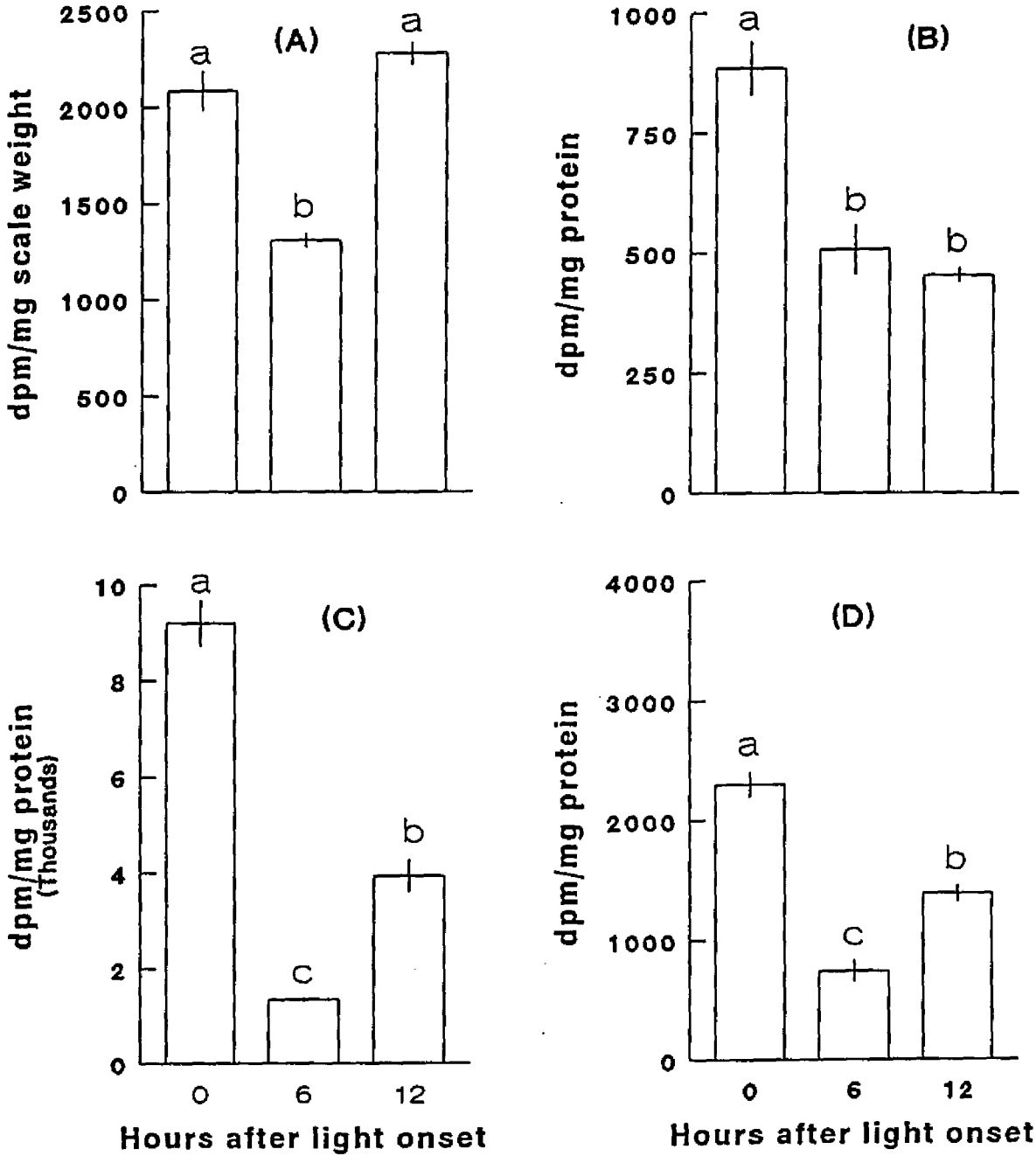


Figure 2. *In vitro* ^{14}C-glycine incorporation by (A) scales (B) muscle (C) liver and (D) intestine of male fish at three different times during a day. Means with the same small case letter are not significantly different from each other ($p > 0.05$, DMRT)

In vivo incorporation of glycine also varied during a day (ANOVA p values : scale, $p < 0.001$; muscle, $p < 0.001$; liver, $p < 0.05$; intestine $p > 0.05$ ns) in female fish (Experiment 3, Figure 3). Glycine incorporation was greatest during late scotophase in scale, early photophase in muscle and early scotophase in liver. Females differed from males most obviously with respect to glycine incorporation by liver and intestine.

The incorporation of the label in response to growth hormone and prolactin varied with time-of-day of hormone administration, and with the interval between hormone administration and assay (Figure 4a-c). Growth hormone injection at the onset of light resulted in an increase in glycine incorporation 3 h ($p<0.01$) and 6 h ($p<0.05$) after hormone treatment (Figure 4a). Injection of growth hormone 6 h after the onset of light produced a moderate increase (statistically not significant) in incorporation 3 h after hormone injection (Figure 4b).

The response to prolactin treatment at light onset was similar to that of hormone injection (Figure 4a). A significant increase was found 3 h after hormone administration, and remained significantly different from the saline treated group 6 h after hormone injection. Injection of prolactin 6 h after the onset of light increased incorporation only when assayed 1 h after hormone administration (Figure 4b). The increase appears to be a

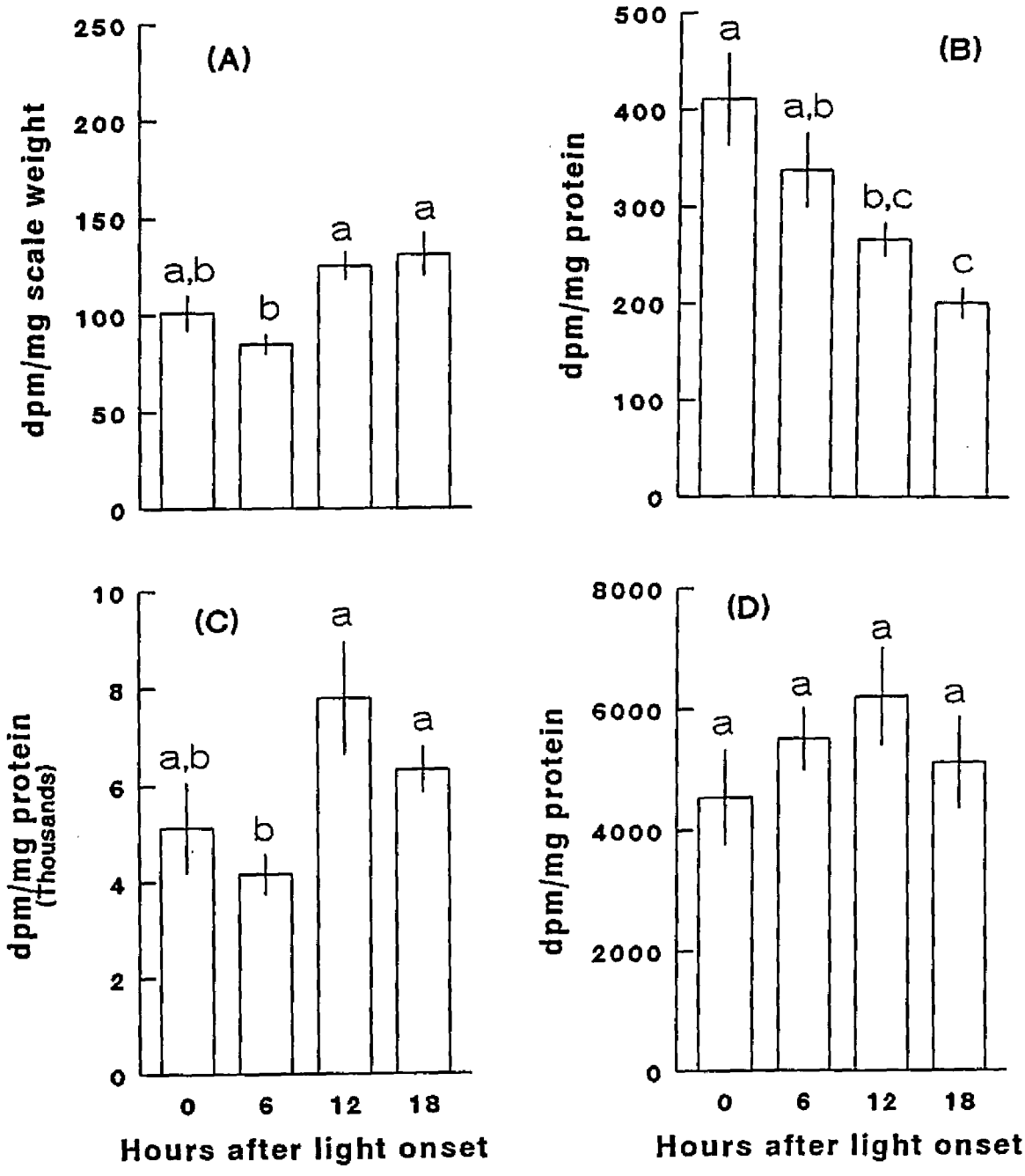


Figure 3. *In vivo* ^{14}C-glycine incorporation by (A) scales (B) muscle (C) liver and (D) intestine of female fish injected with ^{14}C-glycine at four different times during a day. Means with the same small case letter are not significantly different from each other ($p > 0.05$, DMRT)

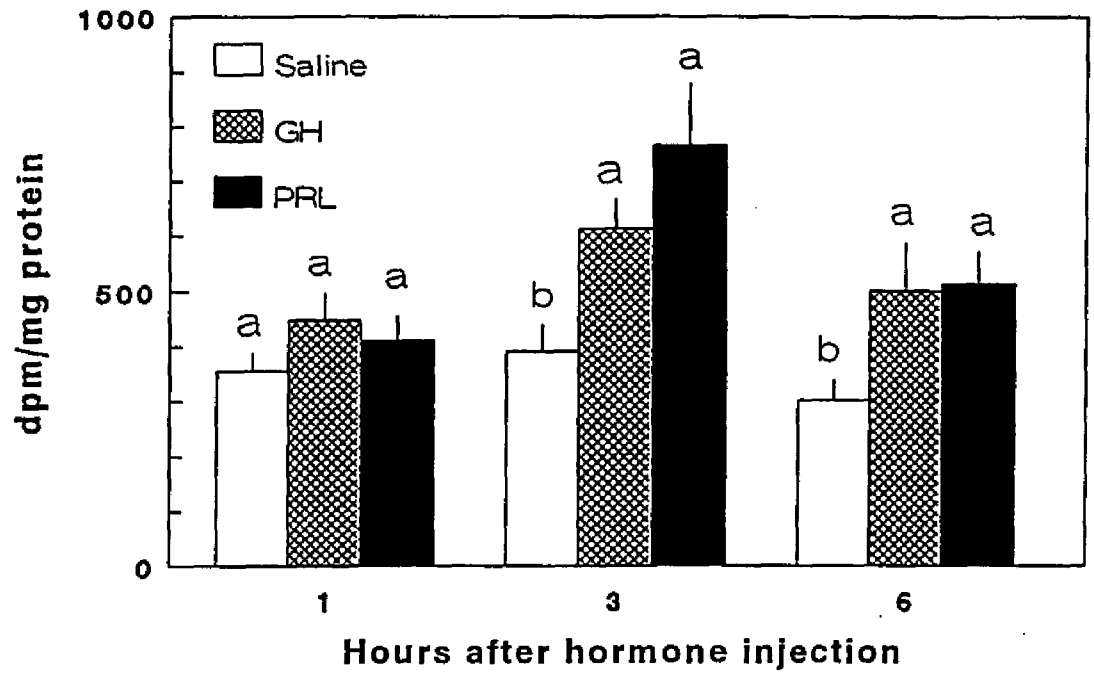


Figure 4a. *In vivo* ^{14}C-glycine incorporation by muscle of fish after 1, 3 and 6 h following injection with saline (control), growth hormone (GH) or prolactin (PRL) at light onset (0800 h). Means with the same small case letter within the same assay time are not significantly different from each other ($p > 0.05$, DMRT)

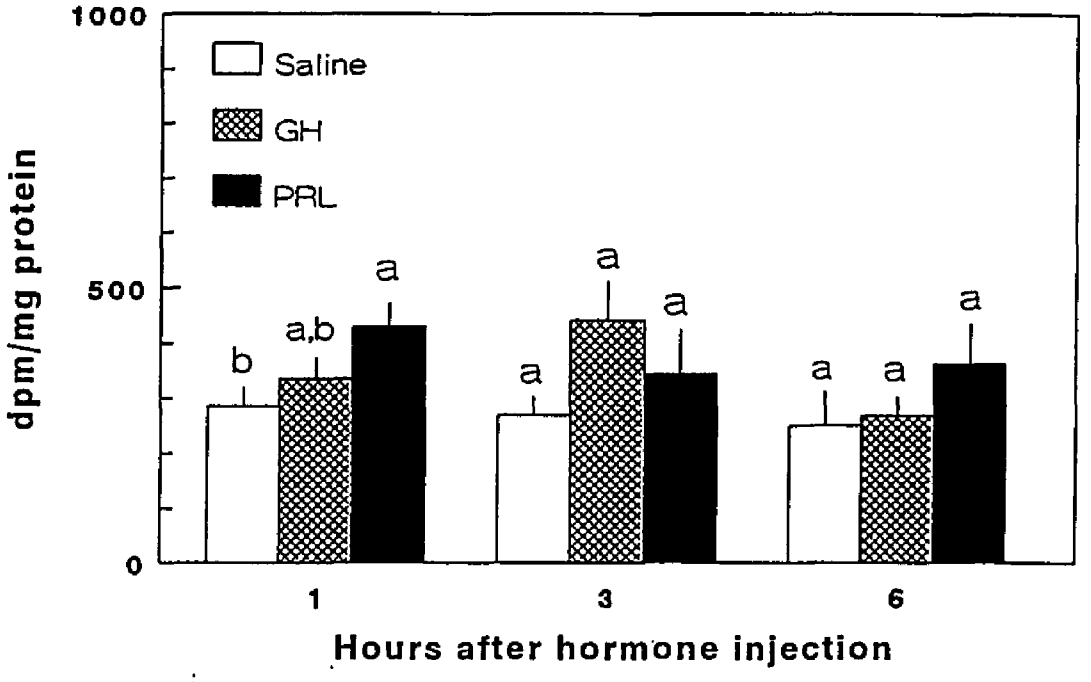


Figure 4b. *In vivo* ^{14}C-glycine incorporation by muscle of fish after 1, 3 and 6 h following injection with saline (control), growth hormone (GH) or prolactin (PRL) 6 h after light onset (1400 h). Means with the same small case letter within the same assay time are not significantly different from each other ($p > 0.05$, DMRT)

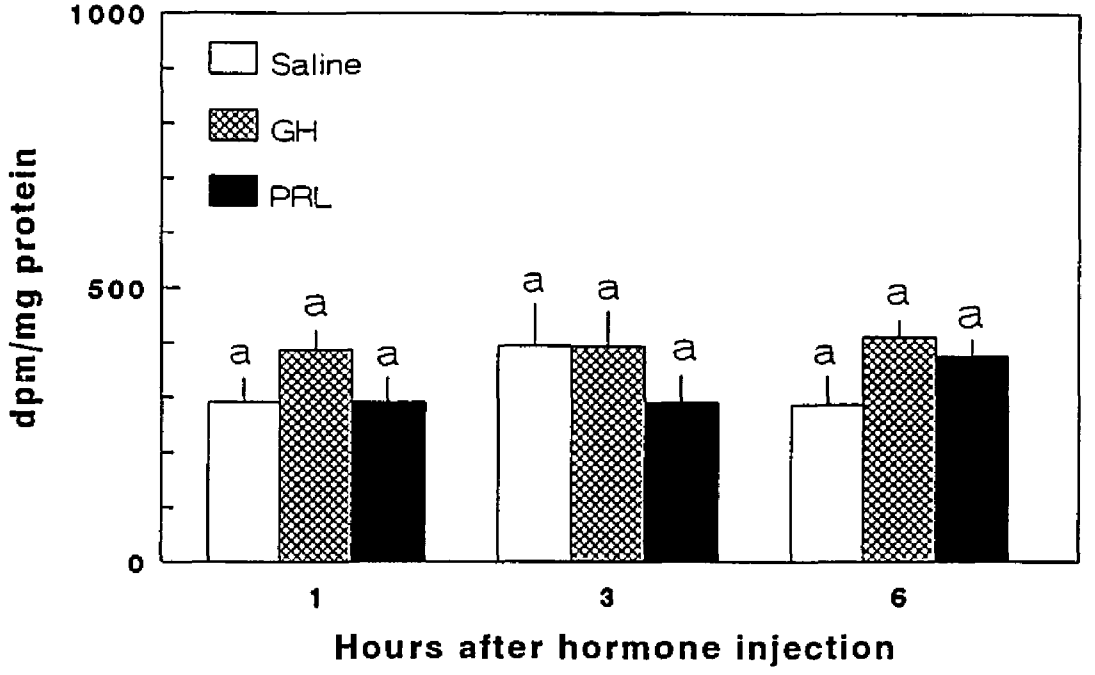


Figure 4c. *In vivo* ^{14}C-glycine incorporation by muscle of fish after 1, 3 and 6 h following injection with saline (control), growth hormone (GH) or prolactin (PRL) at the onset of dark period (2000 h). Means with the same small case letter within the same assay time are not significantly different from each other ($p > 0.05$, DMRT)

transient one since 3 and 6 h post-hormone injection glycine incorporation were not significantly different from the saline injected group. Both hormones were least effective when administered 12 h after the onset of light, that is at the onset of the dark period (Figure 4c).

Discussion

The following generalizations can be drawn from the results of this study. First, there are daily variations in ^{14}C-glycine incorporation, and hence in the rate of protein synthesis, in several tissues. Second, the time of day of peak incorporation is not the same for all tissues. Third, the times of peak incorporations by scales and muscle appeared similar in male and female fish whereas peak incorporation times of liver and intestine appear to differ between the sexes. And fourth, incorporation of labeled glycine into muscle protein in response to growth hormone and prolactin is variable depending on the time of day of hormone injection, and on the time of assay after hormone injection.

Demonstrations of daily variations of protein synthesis in *Fundulus grandis* corroborate other studies that demonstrate daily rhythms of physiological processes and of hormones that influence growth. Circadian variations in protein synthesis in fish have previously been reported for scales of *Rutilus rutilus* (Ottaway, 1978)

and for otoliths of goldfish (Mugiya *et al.*, 1981). Ottaway (1978), who also used ^{14}C-glycine incorporation as a measure of protein synthesis, reported peak glycine incorporation at about the same hour of the day in winter and summer, after the onset of dark in winter (9L:15D) and before the onset of dark in the summer (18L:6D). In the present study carried out on a 12-h daily photoperiod, glycine incorporation by scales occurred mostly during the dark period.

The daily variation in protein synthesis observed in the present and other studies may reflect the rhythms of hormones regulating protein metabolism. It has been shown that systemic hormones (GH, PRL, T_3, IGF-I) which influence protein synthesis undergo daily variations; however, the daily peak values of these hormones often do not occur at the same time of the day. In gold fish, plasma T_4 and T_3 peaks have been reported to occur during the late photophase (Spieler and Noeske, 1979) whereas growth hormone and prolactin peaks occur during the first and second half of the scotophase, respectively (Leatherland and McKeown, 1973; Leatherland *et al.*, 1974; Bates *et al.*, 1989). In *Fundulus grandis*, the plasma Sm-C (IGF-I) peak was found at the onset of light during summer or later during the photophase in winter (Emata, 1990; Wilson *et al.*, 1990). Accordingly, the rhythms of protein synthesis in the liver, which is directly responsive to growth

hormone and prolactin, may well differ from that of muscle or other tissues which are stimulated indirectly by these hormones through IGF-I. Growth hormone and prolactin receptors are numerous in the liver and scarce or absent in many other tissues of fish (Edery *et al.*, 1984; Hirano, 1991; Yao *et al.*, 1991).

The daily variations of protein synthesis and the phase differences of these rhythms in the several tissues examined may also be related to tissue specific rhythms of responsiveness to growth promoting hormones. Several studies demonstrate that there are daily rhythms of tissue responsiveness to hormonal stimuli in teleosts (e.g. Joseph and Meier, 1971; Emata, 1990). Phase differences in response rhythms of two separate tissues to a single stimulus (prolactin) have also been shown previously. The time of a daily peak of fattening response to prolactin in pigeons differs from the time of the daily peak of cropsac response to prolactin (John *et al.*, 1972). The phases of rhythms of lipogenic (liver) and hypoglycemic (mainly muscle) responses to insulin also differ in hamsters (Cincotta and Meier, 1984).

Protein synthesis by muscle and scale closely correlate with the overall body growth of fish. It has been suggested (Haschemeyer and Smith, 1979) that protein synthesis in muscle is the most closely correlated with whole body growth rate of fish because of a low turnover

rate of muscle protein in comparison with other tissues. Furthermore, significant correlation between labeled glycine uptake by scales and growth rate of individual fish have been found for sea bass, *Dicentrarchus labrax* (Ottaway and Simkiss, 1979) and largemouth bass, *Micropterus salmoides* (Smagula and Adleman, 1983). Accordingly, the muscle and scale rhythms seem to be good indices of rhythms (i.e., of stimuli and responses) involved in organismal growth.

Although almost all attention has been given to circadian rhythms of stimuli, such as hormones, it is probable that rhythms of target tissue responsiveness (i.e., sensitivity) to the stimuli are at least as important for determining resultant rhythms and metabolic conditions. For example, the time of day when prolactin was given was much more important than the amount of prolactin given in eliciting a fattening response in *Fundulus grandis* (Meier, 1984). Furthermore, in *Fundulus grandis*, apparently normal rhythms of plasma cortisol persisted after the adrenocorticotropic hormone rhythm was obliterated by hypophysectomy (Srivastava and Meier, 1972). That is, the cortisol rhythm reflects a rhythm of cortical sensitivity/secretion regulated by the autonomic nervous system (Ottenweller *et al.*, 1978; Ottenweller and Meier, 1982). The temporal interaction between a stimulus rhythm and rhythm of responsiveness to the stimulus is thought to

have an important role in endocrine regulation. That is, the greatest net effect is produced when the peak of the daily stimulus coincides with the daily interval of greatest responsiveness to the stimulus. All other phase relations of the stimulus/response rhythms produce a gradation of lesser effects (Meier, 1984).

In this light, it is noteworthy that both prolactin and growth hormone induce much greater increases in plasma concentrations of IGF-I, which mediates most of the growth promoting activities of the pituitary hormones, when prolactin and growth hormone are given at some times of day than at others in *Fundulus grandis* (Emata, 1990). Inasmuch as most plasma somatomedin derives from the liver in many vertebrates (Ooi and Herington, 1988), a temporal interaction of pituitary hormone rhythms with rhythms of hepatic responses to these hormones may be important in the regulation of organismal growth. That is, the greatest amount of somatomedin secretion, and growth, would be expected when the daily peak of the pituitary hormone coincides with the daily interval of greatest hepatic responsiveness. All other phase relations of the stimulus and response rhythms would produce lesser amounts of growth. It seems clear from these initial findings that circadian rhythms may have primary roles in the organization of protein metabolism and that more studies are needed to explore how the interactions of various

hormone rhythms and target tissue response rhythms may regulate growth and development.

CHAPTER 2

In vitro Incorporation of ^{14}C-glycine into Muscle Protein of the Gulf Killifish (*Fundulus grandis*) in Response to Insulin-like Growth Factor-I

Introduction

Insulin-like growth factor-I (IGF-I, somatomedin-C) is a polypeptide known to mediate several actions of growth hormone in mammals (eg. Binoux *et al.*, 1982; Herington *et al.*, 1983; for review: Humbel, 1990). IGF-I accelerates whole body growth (Schoenle *et al.*, 1985; van Buul-Offers *et al.*, 1988) and induces selective growth of several tissues (Guler *et al.*, 1988). It stimulates protein synthesis *in vivo* (Pell and Baites, 1992) and *in vitro* (Ballard *et al.*, 1986; Gulve and Dice, 1989; Fuller *et al.*, 1992). Growth hormone stimulates IGF-I production by cultured rat liver in a dose-dependent manner (Binoux *et al.*, 1982) and increases IGF-I mRNA *in vitro* (Roberts *et al.*, 1986) and *in vivo* (Vikman *et al.*, 1991).

Although early studies (Furlanetto *et al.*, 1977; Wilson and Hinz, 1982) failed to detect IGF-I in fish plasma, several recent studies have demonstrated the presence of immunologically reactive IGF-I in plasma of several teleosts (Daughaday, *et al.*, 1985; Lindahl *et al.*, 1985; Funkenstein *et al.*, 1989; Drakenberg *et al.*, 1989) including gulf killifish (*Fundulus grandis*) (Emata, 1990). The physiological role of IGF-I in teleosts has received comparatively little attention. Studies thus far have been limited to measurements of its effects on whole body growth (Skyrud *et al.*, 1989; McCormick, *et al.*, 1992) and

^{35}S-sulfate uptake in cultured fish cartilage (Duan and Hirano, 1990; Gray and Kelley, 1991). Although IGF-I is known to stimulate protein synthesis in mammals, this possibility has not been explored in fish tissues. The present *in vitro* study examines the effect of recombinant human insulin-like growth factor I (rhIGF-I) on the incorporation of ^{14}C-glycine into the trichloroacetic acid (TCA) precipitable fraction of muscle protein in the gulf killifish, *Fundulus grandis*.

Materials and Methods

Animals. Gulf killifish, *Fundulus grandis*, were obtained from a commercial dealer who collected them from brackish water along the Louisiana coast of the Gulf of Mexico. The fish were held on 12-hour daily photoperiods for more than a month prior to experimentation. The water was maintained at a temperature of 24 $\pm$ 1°C and the salinity (Instant Ocean) was held at 5‰. The fish were fed commercial fish food (Tetramin) two to three times a day at variable times during the photophase. With the exception of Experiment 5, all experimental fish were sacrificed with an overdose of MS-222 during the first hour after the onset of light.

Tissue preparation and incubation. Muscle tissue was taken from the lateral sides of the caudal region and minced into 1 mm thick slices using a McIlwain tissue

chopper. Each slice weighed about 15 mg and had a surface of nearly 2 by 5 mm (based on measurements of tissue slices not used in the experiments). In each experiment, an equal number of tissue slices taken from each fish were pooled from several fish (10-15 fish). The slices were washed with buffered Minimum Essential Medium (MEM, Sigma; pH 7.4) supplemented with penicillin (1000 U/ml), streptomycin (100 μg/ml) and 20 mM HEPES. The pooled tissue slices were equally divided among the groups in each experiment. Incubation was at room temperature (24°C) in Falcon petri dishes, each containing 5 ml of MEM. In all the experiments, except Experiment 2, the amount of incorporation of labeled glycine was determined after incubating the tissues for 1 h in 5 ml medium containing 4 μCi/ml ^{14}C-glycine (ICN Biochemicals, specific activity of 52 mCi/mmol). Labeled glycine (in 0.01N HCl) was diluted with incubating medium sufficient for each experiment. Following incubation in labeled glycine the tissue slices were transferred into 25 ml Erlenmeyer flasks, washed by gentle agitation with 3 changes of 5 ml fish saline (Shuttleworth, 1972 : composition in mmol/l are: NaCl 136.9; KCl 2.7; $CaCl_2$ 2.98; $NaHCO_3$ 14.99; NaH_2PO_4 3.17) and immediately frozen for later determinations of protein and radioactivity.

Experiment 1. Muscle slices were divided into six groups. Five groups were incubated in MEM without

^{14}C-glycine for variable times (4, 8, 12, 16 and 20 h). The medium was replaced with fresh medium every 4 h. Following the variable incubation times, the medium was removed and replaced with fresh medium containing labeled glycine and incubated for another 1 h to ascertain uptake of label. One group (0 h group) was incubated directly in medium with labeled glycine for 1 h.

Experiment 2. Tissue slices were divided into three groups. The slices were incubated in MEM containing ^{14}C-glycine alone (without inhibitor) (group 1), with actinomycin D (20 μg/ml) (group 2) or with cycloheximide (20 μg/ml) (group 3). The medium was changed every 5 h. For each treatment group the amount of label incorporated was determined at 5, 10, 15 and 20 h of incubation with label present.

Experiment 3. Dose response. Recombinant human insulin-like growth factor-I (rhIGF-I, Calbiochem) in concentrations of 0 to 100 ng/ml of incubation medium (MEM) was used. Muscle tissue slices were divided into six groups. Following a preincubation of 3 h in 5 ml medium the tissues were incubated for 10 h in fresh medium containing 0, 0.01, 0.1, 1.0, 10.0 or 100 ng/ml rhIGF-I. The medium was changed once at 5 h of incubation. Then the medium was replaced with a fresh medium containing ^{14}C-glycine, incubated for another 1 h and the tissue was

frozen for later determinations of protein content and glycine incorporation by the tissue.

Experiment 4 - rhIGF-I Time-course effect. Muscle slices were divided into two groups (controls and IGF-I treated). Controls were incubated in medium (MEM) only and experimentals were incubated in medium containing 1 ng/ml rhIGF-I for 3 h, then the medium was replaced with fresh medium without rhIGF-I for both controls and experimentals. Starting at the end of the 3 h treatment period and thereafter at 3 h intervals (3,6,9,12 and 15 h) one subgroup from each of the controls and IGF-I treated groups was removed, incubated for 1 h in medium containing ^{14}C-glycine and the tissue frozen for later analyses of protein content and glycine incorporation.

Experiment 5 - Time-of-day effect. At six-hour intervals during a day beginning at light onset, fish were sacrificed to obtain muscle slices (seven fish at each time). Muscle slices from each fish were divided equally into two groups. One group (control) was incubated in MEM alone and the other in MEM containing 1 ng/ml IGF-I. After three hours the medium was replaced with fresh medium without IGF-I for both groups. Incubation was continued for another three hours and then labeled glycine was added to the medium, incubated for 1 h and the tissue frozen. In this experiment incubation with hormone was restricted to 3 h so that the time of hormone treatment was closer to the

time of tissue removal. The 3 h incubation following treatment with IGF-I was required since greater response to hormone was found 6 h after onset of 3 h hormone treatment (see result of Experiment 4).

Protein and radioactivity determinations. Tissue slices were homogenized in 1 ml Tris (pH 7.4), centrifuged for 5 min at 1000 xg, and the pellet discarded. One ml of 10% TCA was added to the supernatant, left for 1 h on ice, and centrifuged at 4300 xg for 30 min. The pellet was washed with 100 mM unlabeled glycine, air dried, and resuspended in 0.2 ml 1 N NaOH. 5 μl aliquots were used for protein determinations by the method of Lowry, *et al.* (1951), using bovine serum albumin (Sigma) as a standard. 100 μl aliquots were used for the determination of radioactivity using a Beckman 8000 liquid scintillation spectrophotometer. ^{14}C-glycine incorporation is expressed as dpm/mg protein.

Data analyses. Data were analyzed by analysis of variance (ANOVA). Where differences were significant ($p<0.05$) the means were compared by Duncan's multiple range test (DMRT).

Results

Experiments 1 and 2. These experiments were undertaken to determine whether muscle tissue incorporates labeled glycine into protein during incubation. The

results (Table 1 and 2) indicate that protein synthesis occurs throughout a 20 h incubation period. Actinomycin D and cycloheximide greatly reduced protein synthesis (Table 2). After 5 h of incubation actinomycin D reduced glycine incorporation by 24% and cycloheximide reduced it by 71% compared with the control. At the end of 20 h incubation the reduction in glycine incorporation was 74% and 92% in actinomycin D and cycloheximide treated media, respectively. Curiously, the rate of ^{14}C-glycine incorporation apparently increased gradually with increasing period of incubation that occurred before adding labeled glycine (Table 1). Inasmuch as the incubation medium contained no unlabeled glycine the prolonged incubation may have progressively depleted the cytoplasmic pool of glycine. This would result in an increasing ratio of labeled to unlabeled glycine and a greater uptake of radioactivity as the incubation period was prolonged.

Tissue slices have also been processed for histological examination in order to assess any change that may have occurred during the prolonged period of incubation. Observation of tissues that were fixed and stained after incubation for 10 or 20 h showed no apparent changes in appearance when compared to the unincubated tissue preparation (Figure 5). However, tissue slices that were incubated for 20 h before fixation showed minor patches of necrotic areas. The nuclei of the satellite

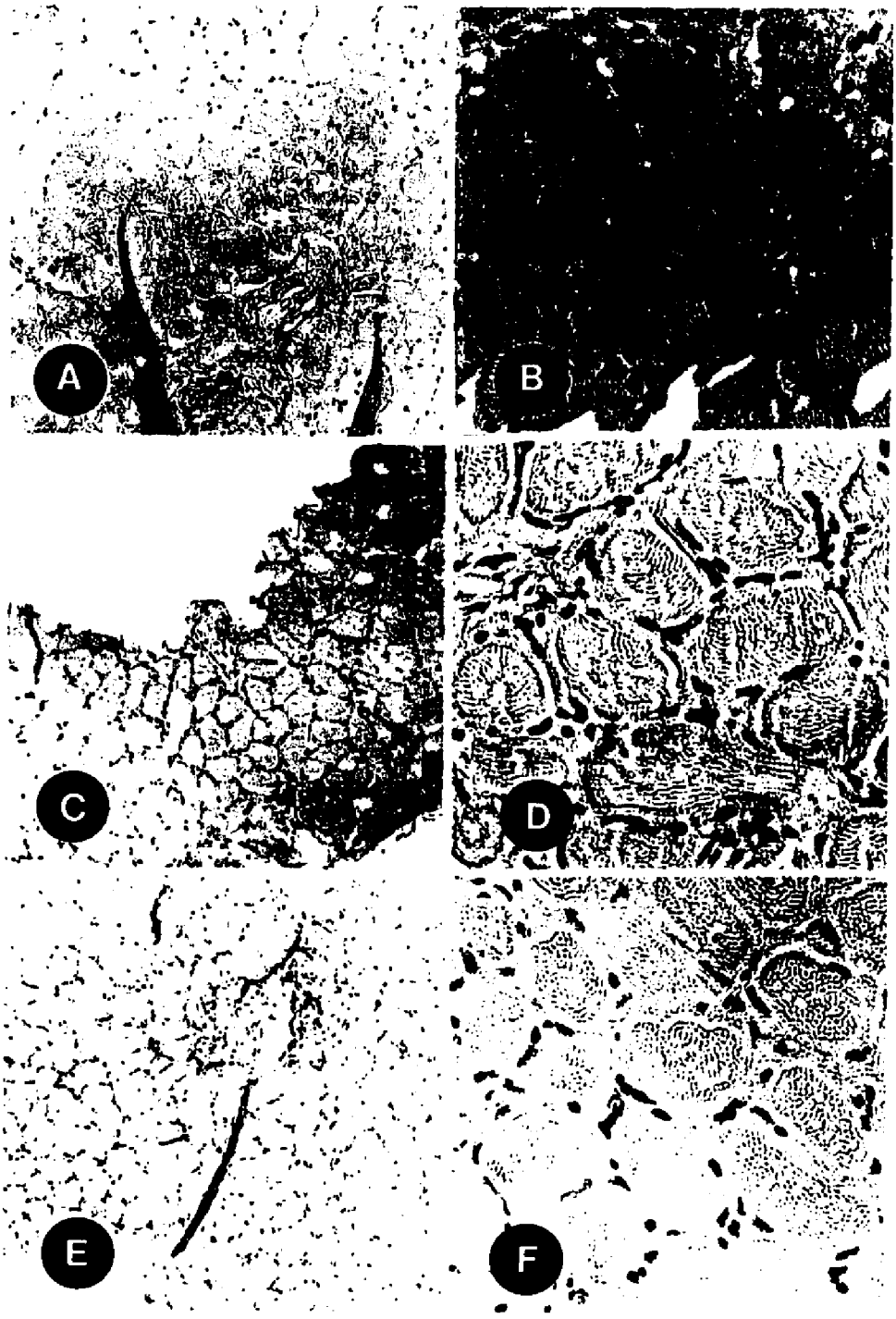
Table 1 - ^{14}C-glycine incorporation in tissue incubated in MEM for different periods before adding labeled glycine. Values are means ± S.E.M., n=6. Means with the same superscript letter are not significantly different ($p > 0.05$, DMRT).

Incubation time (h)	Incorporation (dpm/mg protein)
0	340.9 ± 26.1^{c}
4	$571.5 \pm 40.3^{b,c}$
8	407.3 ± 27.0^{c}
12	758.8 ± 20.1^{b}
16	1380.9 ± 218.9^{a}
20	1580.9 ± 57.9^{a}

Table 2 - ^{14}C-glycine incorporation in tissue incubated for different periods in the presence or absence of inhibitors: actinomycin D or cycloheximide. Values are means ± S.E.M. Means with the same superscript letter in the same row are not significantly different from each other ($p > 0.05$, DMRT).

Incubation Period (h)	Incorporation (dpm/mg protein)		
	Control	Actinomycin D	Cycloheximide
5	1554.4 ± 35.1^a	1177.2 ± 11.8^b	453.2 ± 25.1^c
10	4949.9 ± 75.1^a	1890.2 ± 24.1^b	706.4 ± 14.2^c
15	9633.5 ± 34.6^a	2683.3 ± 30.6^b	1443.0 ±515.5^c
20	18906.5 ± 278.6^a	4884.5 ± 125.2^b	1525.4 ± 37.8^c

Figure 5. Cross section of muscle fixed immediately after dissection (A,B), or after incubation in MEM for 10 h (C, D) or 20 h (E,F). Tissues were fixed in 10% neutral buffered formalin for 72 h and stained with hematoxylin and eosin following the staining procedure of Luna (1968). Tissues were sectioned at 5 μm thickness. Magnification: left panel 100x; right panel 400x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cells, which account for the incorporation of the labeled glycine, appear to be normal in their appearance.

Experiment 3. Treatment with rhIGF-I resulted in an increase in ^{14}C-glycine incorporation (Figure 6). Comparison of the means shows a significant difference (ANOVA $p<0.001$). A significant increase was achieved at 0.1 ng/ml and maximum glycine incorporation was observed at a concentration of 1 ng/ml. Glycine incorporation declined with rhIGF-I concentrations greater than 1 ng/ml. 10 ng/ml and 100 ng/ml rhIGF-I were completely ineffective in changing glycine incorporation.

Experiment 4. Figure 7 illustrates the time-course effect of rhIGF-I on ^{14}C-glycine incorporation. At the end of the 3 h treatment with rhIGF-I (1 ng/ml) there was a significant increase in glycine incorporation ($p<0.01$) compared to the control. The greatest response was found 6 h after onset of 3 h rhIGF-I treatment. The response 9 h after the onset of 3 h treatment was also significant but of a lower magnitude. Twelve and 15 hours after the onset of 3 h rhIGF-I treatment there were no significant differences in incorporation.

Experiment 5. The effect of time of day of treatment with 1 ng/ml of rhIGF-I on ^{14}C-glycine incorporation is presented in Figures 8a and 8b. The results indicate that tissue taken at different times of the day did not exhibit equivalent responses to rhIGF-I. A major increase in

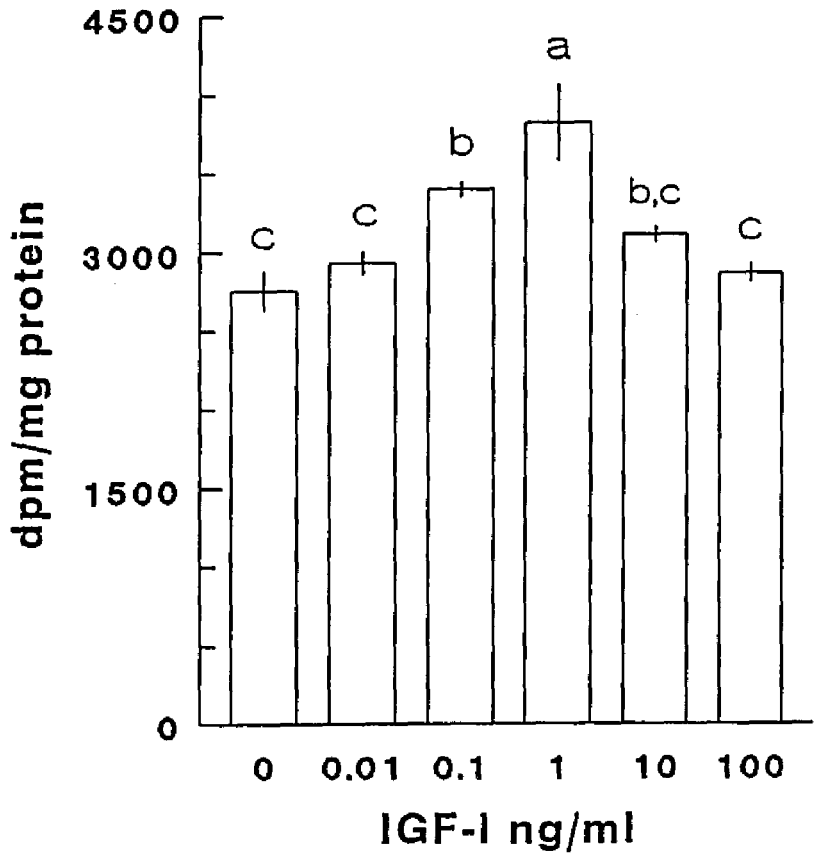

Figure 6. Effects of different concentrations of rhIGF-I on ^{14}C-glycine incorporation. Values represent means ±S.E.M. of replicate samples (n=5). Values with the same lower case letters are not statistically different from each other ($p > 0.05$ DMRT).

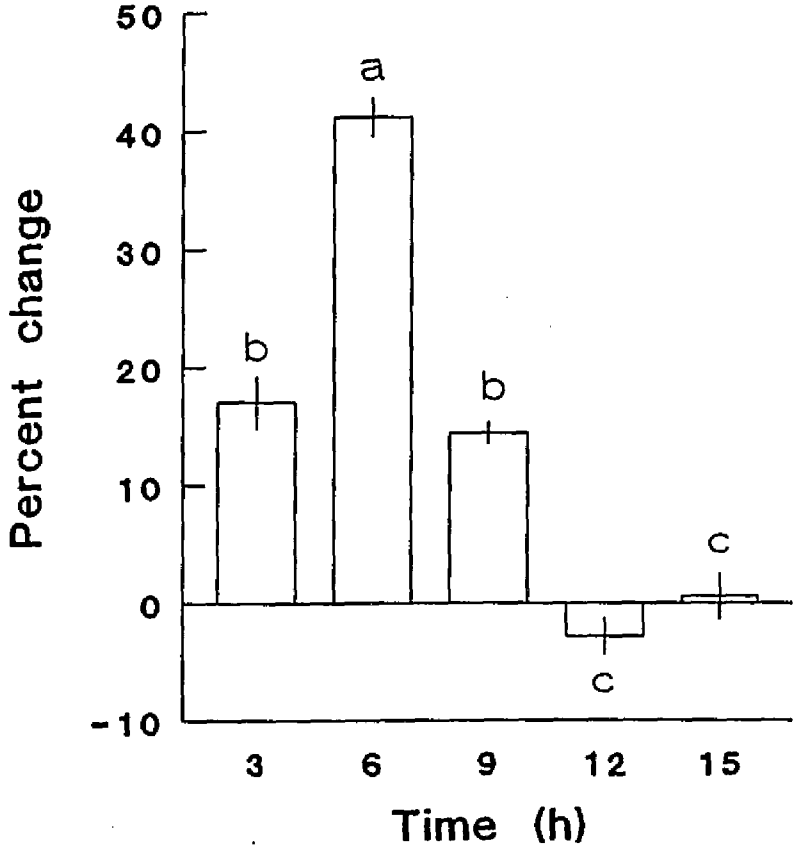

Figure 7. Time-course effect of IGF-I on ^{14}C-glycine incorporation. Values represent percent changes from the corresponding controls. Values with the same lower case letter are not statistically different from each other ($p > 0.05$ DMRT).

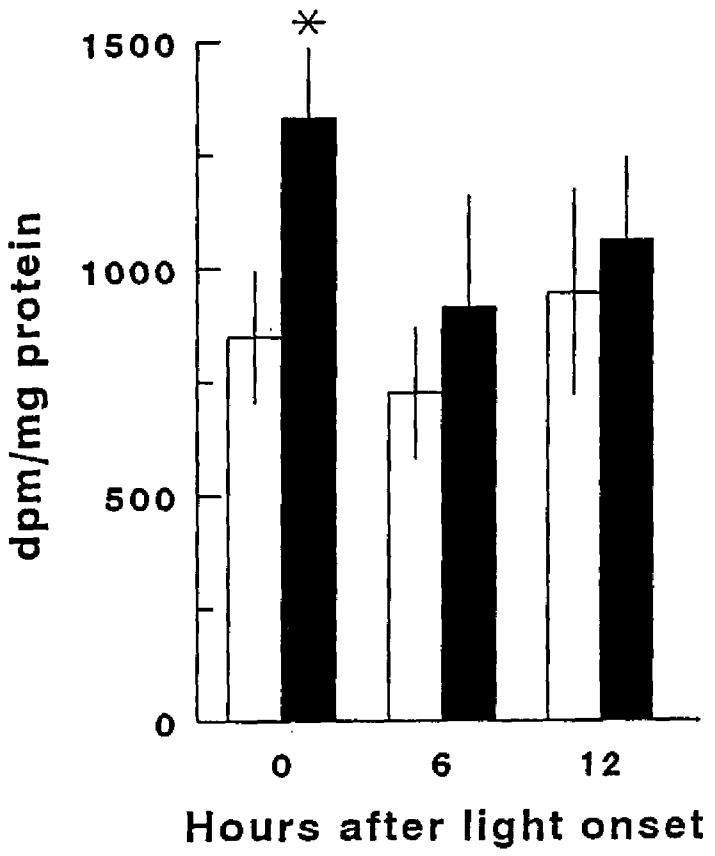

Figure 8a. Tissue response to rhIGF-I at different times of day (September). Open bar= control, solid bar = rhIGF-I treated. Values are means ± S.E.M. of 6 fish. Data were analyzed by one-way ANOVA on the differences between treated and control. * $p<0.05$ compared with the corresponding control.

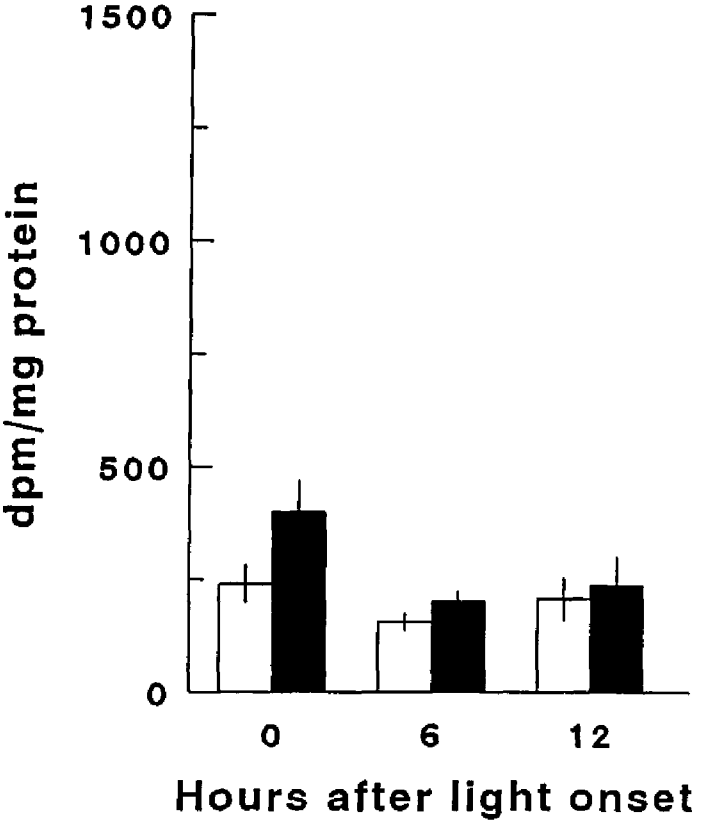

Figure 8b. Tissue response to rhIGF-I at different times of day (February). Open bar= control, solid bar = rhIGF-I treated. Values are means ± S.E.M. of 7 fish. Data were analyzed by one-way ANOVA on the differences between treated and control.

glycine incorporation in response to rhIGF-I was observed in tissues that were taken at light onset (0800 h). A significant ($p=0.03$) response at light onset was found in the experiment performed in September (Figure 8A). In the experiment done in February (Figure 8B) the difference was not statistically significant; however the response at light onset tended to be higher than at other times of day studied. Pooled data of the two experiments showed a highly significant ($p<0.01$) response at light onset. Tissues taken at other times of day (6 and 12 h after light onset) were not as responsive to the treatment. The observed difference in magnitude between the two experiments may be due to seasonal differences in growth rates. In gulf killifish maintained under constant laboratory conditions it has been observed that growth rate is greater in summer than in winter (Emata, 1990).

Discussion

Sliced muscle preparation was used to investigate the effect of rhIGF-I on the incorporation of ^{14}C-glycine into the TCA precipitable fraction of fish muscle. Such tissue preparations have been used by other investigators for *in vitro* studies (eg. Bresnick and Burki, 1975; Binoux, *et al.*, 1980; Inui and Ishioka, 1985). In intact muscle used for *in vitro* studies histological observations show that the more central part of the tissue undergoes atrophy

(Harris, *et al.*, 1985). The use of thin tissue preparations helps to minimize this effect since this facilitates the diffusion of substances to and from the core of the tissue. In the present study the results of Experiments 1 and 2 (Tables 1 and 2) and histological examinations (Figure 5) demonstrate the viability of cells during the period of incubation. The reduction in glycine incorporation in the presence of actinomycin D or cycloheximide (Table 2) indicate that newly synthesized RNA and protein synthesis are required to maintain the high rate of incorporation of the label.

^{14}C-glycine incorporation increased in a dose-dependent manner at lower doses of rhIGF-I (<1 ng/ml) (Figure 6). At concentrations greater than 1 ng/ml the response declined to control levels. The parabolic nature of the dose-response to IGF-I is also evident in the studies by Duan and Hirano (1990) and Gray and Kelley (1991) who reported decreased sulfation responses of fish cartilage at concentrations greater than 10 and 250 ng/ml, respectively, for the preparations they used. Decreased responses at higher doses may result from down-regulation of IGF-I receptors. It has been shown that preincubation of mammalian tissues with IGF-I down-regulates the IGF-I receptors (de Vroede, *et al.*, 1984), probably by a decrease in receptor number (Rechler and Nissley, 1985; Yamamoto, *et al.*, 1993).

The increased glycine incorporation in response to rhIGF-I may be the result of enhanced amino acid uptake and/or a stimulatory effect on protein synthesis. A recent report by Duclos, *et al.* (1993) shows that IGF-I stimulates protein synthesis and promotes amino acid uptake. This effect can be reduced when protein synthesis is inhibited with cycloheximide. The time course for glycine incorporation in the present study (6 h for maximal stimulation by rhIGF-I, Figure 7) favors a more direct stimulation of protein synthesis by IGF-I.

Distinct high affinity receptors for insulin and IGF-I have been demonstrated in many different tissues (Beguinot, *et al.*, 1985). However, because of the structural similarities between IGF-I and insulin and between their receptors (Humbel,1990), IGF-I and insulin interact with each other's receptor and have some activities in common, however, this interaction is concentration-dependent such that one interacts substantially with the other's receptor but with less binding affinity when present at high concentrations (Gammeltoft, *et al.*, 1985). Accordingly IGF-I enhanced protein synthesis (incorporation of labeled glycine into protein) most likely through its own receptor rather than an interaction with the insulin receptor.

Although much less studied in fish than in mammals, several studies in teleosts also suggest that IGF-I may be a mediator of the actions of growth hormone. Circulating

plasma immunoreactive somatomedin-C has been demonstrated in several teleost species (Lindahl *et al.*, 1985; Drakenberg *et al.*, 1989; Funkenstein *et al.*, 1989) and increases in levels of plasma immunoreactive somatomedin-C occur in response to bovine growth hormone injection in the gulf killifish (Emata, 1990; Wilson, *et al.*, 1990). Inui and Ishioka (1985) reported that growth hormone increased protein synthesis (incorporation of ^{14}C-leucine) in opercular muscle of the eel *in vivo* with little (in liver) or no (in muscle) effect *in vitro*.

It should be noted that there are also negative results for IGF-I actions in fish. Injections of brook trout with human IGF-I failed to promote growth (Skyrud *et al.*, 1989); IGF-I decreased body weight and length at doses of 1 and 3 μg/g body weight; at a dose of 10 μg/g body weight, IGF-I resulted in the death of fish. But in fish receiving a dose of 0.1 μg/g body weight, body weight and length remained at the control level. Unfortunately, lower doses were not used. In a similar study (McCormick, *et al.*, 1992), recombinant bovine IGF-I injections increased growth of coho salmon at a lower dose but doses greater than 0.13 μg/g resulted in hypoglycemia and death of the fish. Inasmuch as IGF-I cross reacts with insulin receptors (Humbel, 1990) and mammalian insulin causes hypoglycemia in fish (Ablett, et al., 1981; Carneiro and Amaral, 1983), the higher (probably nonphysiological) doses

used may have favored greater interaction with insulin receptors leading to hypoglycemia, as well as down-regulation of IGF-I receptors leading to masking of growth promoting effects.

The daily variation in response is construed as indicating a daily variation of tissue sensitivity to the stimulus. Such daily variation of tissue responsiveness in fish has been reported for the effects of prolactin on lipid synthesis (Horseman and Meier, 1979a) and of growth hormone and prolactin on somatomedin-C release (Emata, 1990). Moreover the time of peak responsiveness to a stimulus may differ between tissues. For example, in pigeons, the time of a daily peak of fattening response to prolactin differs from the time of the daily peak of cropsac response to prolactin (John, *et al.*, 1972). Although daily rhythms of hormones may be expected to drive rhythms of metabolism, daily rhythms of tissue responsiveness (ie. sensitivity to stimuli) add an additional important dimension for regulation (Meier, 1984 and 1992). According to this concept a stimulus (hormone) produces its greatest effect when the peak of a stimulus rhythm coincides with the daily interval of greatest responsiveness of the target tissue to the stimulus. Other temporal relations of the stimulus and response rhythms will produce a graded series of lesser responses. Accordingly, a study simultaneously examining daily rhythms

of growth hormone, of somatomedin response to growth hormone, of plasma IGF-I and of responses of several tissues to IGF-I would be useful in exploring a temporal dimension in endocrine regulation of growth.

Although additional studies with IGF-I obtained from fish seem desirable, the present studies and others favor an important mediating role for somatomedins in teleostean growth and protein synthesis. Both dose and time of day are important variables that should be considered in somatomedin activities.

CHAPTER 3

Daily Variation in the Binding of Insulin-like Growth Factor-I in Muscle of the Gulf Killifish, *Fundulus grandis*

Introduction

Changes in the phase relationships of circadian neuroendocrine rhythms are thought to have an important role in the regulation of metabolism (Meier, 1984 and 1992). This temporal interaction may be one involving rhythms of a stimulus and a response to the stimulus. Thus, a stimulus will produce the greatest net effect when the daily peak of the stimulus coincides with the period of greatest responsiveness of tissue to the stimulus. Other temporal relations between the two rhythms will have lesser effects or may even have no effect at all.

Circadian rhythms of stimuli and their direct physiologic or behavioral expressions have received considerable attention (Ali, 1992). Relatively less studied, but regarded equally important in the regulation of biological processes, is the rhythm of tissue-responsiveness to stimuli. Daily variations of tissue responsiveness may involve changes at the receptor level. These may be changes in the rates of receptor synthesis and degradation and/or changes in the membrane properties that can modulate the affinity and accessibility of surface receptors to ligand binding (Shinitzky 1985). Daily variations in receptor numbers and affinities have been demonstrated for the membrane receptors of insulin (Cincotta and Meier, 1985), serotonin (Hulihan-Giblin, *et*

al. 1993), epidermal growth factor (Scheving, *et al.* 1989) and melatonin (Gauer, *et al.* 1993), and the intracellular receptors for estrogen and androgen (Francavilla, *et al.* 1986). Post-receptor events or other cellular processes may also contribute to the daily variation of tissue responsiveness.

In this study the presence of IGF-I receptors in muscle cells of the gulf killifish was examined using recombinant human ^{125}I-IGF-I. The specificity of the receptors to IGF-I, and the daily variations in the binding of ^{125}I-IGF-I were also investigated. Scatchard analysis was used to determine changes in the number and affinity of IGF-I receptors.

Materials and Methods

Animals. The source and maintenance of fish were as described in earlier chapters. Animals were sacrificed with an overdose of MS-222. Except for Experiments 3 and 5, fish were sacrificed at the onset of light (LD 12:12, light onset at 0800 h)

Buffers and ligands. The composition of the buffers used was adapted from published protocols. All buffers were prepared in 0.05 M Tris-HCl with a pH of 7.7 (Blazer-Yost, *et al.* 1989; Cara, *et al.* 1990). The buffers used were: homogenizing buffer [0.25 M sucrose, 5 mM $MgCl_2$, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (Sasaki, *et al.*

1991; McFarland, *et al.* 1992)]; resuspending buffer (5 mM $MgCl_2$, 0.1 mM PMSF) and binding buffer (5 mM $MgCl_2$, 0.25% BSA, 0.1 TIU/ml aprotinin (Cara, *et al.* 1990; Sasaki, *et al.* 1991). ^{125}I-IGF-I (2000 mCi/mol, Amersham) was resuspended in 0.1 M acetic acid and stored at -20°C. Aliquots were diluted in binding buffer just before use. Unlabeled IGF-I (receptor grade recombinant human IGF-I, Mallinckrodt) was resuspended in 100 μl of 0.1 mM acetic acid and stored at -20°C. Further dilutions were prepared in 0.05 M Tris-HCl buffer, pH 7.7.

Tissue preparation. Muscle was dissected from the lateral sides of fish and membrane homogenates were prepared. In all experiments, except Experiment 3, membrane homogenates were prepared immediately after dissection. Tissue, either freshly dissected or stored frozen, was minced with scalpel and placed in 25 ml of homogenizing buffer. The tissues were homogenized intermittently (3 to 4 bursts) for about 30 sec with a Tekmar tissue homogenizer at a setting of 70. The resulting homogenate was centrifuged at 2000 xg for 10 min at 4°C. The pellet was briefly (5 sec) rehomogenized in 5 ml buffer, centrifuged and the supernatant combined with the first supernatant. The pooled supernatant was passed through a 60 mesh sieve screen (Sigma) and centrifuged at 37,000 xg for 15 min. The resulting pellet was resuspended in resuspending buffer. Total protein concentration of the

homogenate was determined by the method of Lowry, *et al.* (1951) using bovine serum albumin as a standard. The protein concentration of the membrane preparations was adjusted with binding buffer.

Binding assay. The binding assay was performed in polypropylene tubes (12 X 75 mm) in a volume of 0.3 ml consisting of binding buffer, membrane preparation, labeled peptide, and unlabeled peptide for determining nonspecific binding. The amounts of membrane homogenate used were 400 μg protein/tube for Experiments 1-3 and 500 μg protein/tube for Experiments 4 and 5. Nonspecific binding was determined in the presence of 300 ng unlabeled IGF-I. Incubation was carried out in a water bath at 20°C for 1 h (except the time-course assay) and the binding reaction was terminated by adding 0.5 ml cold binding buffer. After the addition of the cold buffer the tubes were immediately centrifuged at 4300 xg for 25 min and the supernatant was aspirated. 0.8 ml of the binding buffer was added to the pellet and recentrifuged at 4300 xg for 5 min. This washing step was repeated twice. After the last wash radioactivity was counted using a Beckman 5500 gamma counter.

Experiment 1 - Effect of incubation interval. Muscle was dissected from 10 fish with a mean body weight of 5.86 ±0.11 g (S.E.M.). Membrane homogenates were prepared and the final pellets were pooled for the binding assay. The

binding reaction was carried out for 3 h. Bound radioactivity was measured at 15 min intervals for 1 h and then at 1 h intervals for 2 h.

Experiment 2 - Effect of Tris pH 3.5. A membrane preparation pooled from 10 fish (5.68 ±0.12 g) was used for this study. The pooled preparation was divided into six parts. One part was resuspended in Tris pH 7.7 (control) and the others (treatments) were resuspended and incubated in Tris pH 3.5 for 10, 20, 30, 40 and 60 min. Following incubation in Tris pH 3.5, 3 ml of Tris pH 7.7 was added to each tube (treatments) and centrifuged. The pellets were resuspended in Tris pH 7.7 for the binding assay.

Experiment 3 - Time-of-day effect. A total of 30 fish (body weight 4.18 ±0.03 g) were used for this study. At 4 h intervals during a day, beginning at light onset, 5 fish at each time were sacrificed. Muscle was removed and immediately frozen in liquid nitrogen and stored at -60°C until processing. Membrane homogenates were prepared separately for tissue of each fish. The homogenates were preincubated in Tris pH 3.5 for 10 min (based on results of Experiment 2) and centrifuged. The treated preparations were used for the binding assay.

Experiment 4 - Displacement of tracer by unlabeled IGF-I or insulin. Tissue was collected from 15 fish (5.67 ±0.19 g) and processed to get membrane pellets. The pellets were pooled for the binding assay. Binding was

carried out in the absence (initial binding) or presence of increasing concentrations (10^{-12}-10^{-7} M) of unlabeled IGF-I or bovine insulin (Sigma).

Experiment 5 - Saturation binding assay. At 3 different times during a day (light onset: 0800 h; 6 h after light onset, 1400 h; and 20 h after light onset, 0400 h) groups of 12 fish each were sacrificed. Membrane homogenate of tissue from each fish was prepared separately. At each time homogenates from 4 fish were pooled to obtain 3 samples (groups). ^{125}I-IGFI binding was determined in the presence of increasing concentrations of unlabeled IGF-I.

Data analyses. Binding parameters were determined by Scatchard analysis using a curve-fitting computer program (Equilibrium Binding Data Analysis and Ligand). An F-test comparison of the sum of squares, according to Murray and Siebenaller (1987) was used to determine whether a one- or two-site model fit the data better. One-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) was used to test for differences. Probabilities of 0.05 or less were considered significant.

Results

Incubation time. The time course binding of ^{125}I-IGF-I shows that binding equilibrium is reached near 30 min of incubation (Figure 9). Except for the high binding at

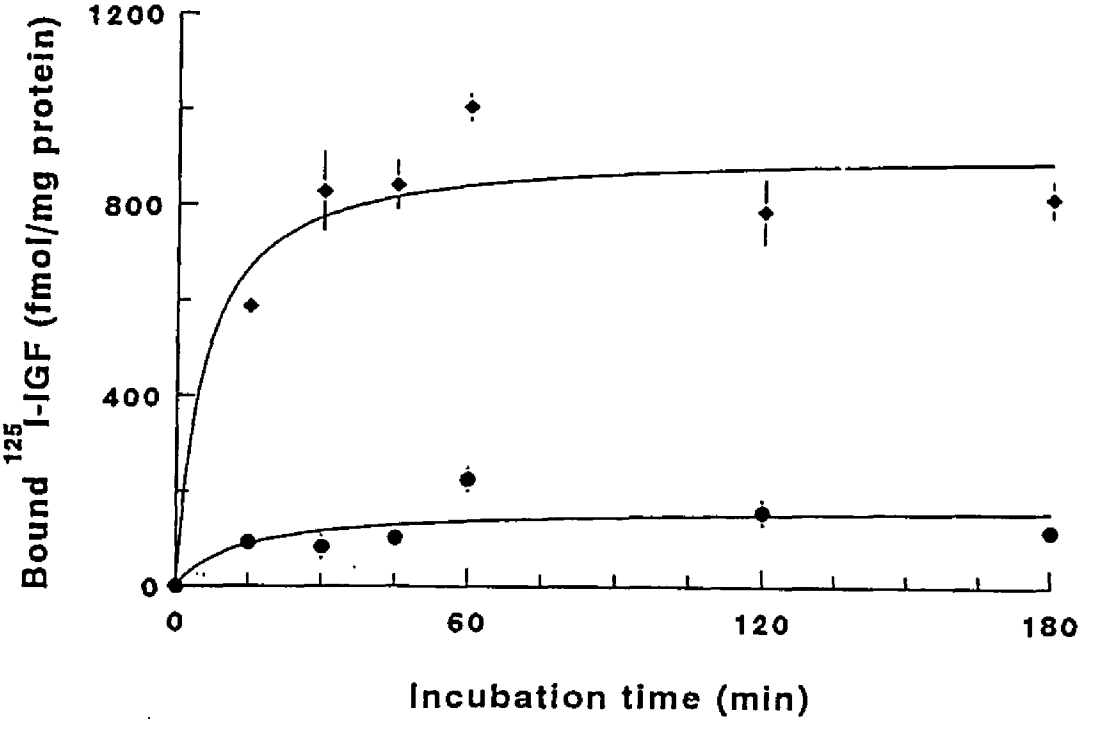

Figure 9. Specific (♦) and nonspecific (●) binding of ^{125}I-IGF-I incubated for different lengths of time. Values are means ±SEM of 3 replicates. Only one determination was made at 15 min.

60 min, the binding remained nearly stable for the next 2 h. In subsequent experiments the binding reactions were run for 1 h.

Preincubation with Tris pH 3.5. The efficacy of 4M $MgCl_2$ and acidified Tris (pH 3.5) in stripping off endogenously bound IGF-I was tested in a preliminary study in which membrane preparations were pretreated with either 4M $MgCl_2$ or Tris pH 3.5 for 10 min before the binding assay. It was found that treatment with 4M $MgCl_2$ greatly reduced binding whereas Tris pH 3.5 increased binding (Figure 10A). The efficacy of Tris pH 3.5 was further tested by treating membrane preparations for varying lengths of time. Pretreatment of tissue homogenates with Tris pH 3.5 greatly increased bound radioactivity (Figure 10B, ANOVA $p<0.001$). Treatments as short as 10 min have a profound effect. A highly significant ($p<0.001$) increase is found in preparations preincubated for 10 min in Tris pH 3.5. An abrupt decrease in binding is noted in pretreatments carried out for 30 min. But at all other times binding, both total and specific, is well above the value for the untreated membrane preparation.

Daily variation. The results of testing the daily variation of binding determined at 4 h intervals is presented in Figure 11. The results show that there is a daily variation in the binding of labeled IGF-I. Lowest binding (860 $\pm$128 fmol/mg protein) was observed at light

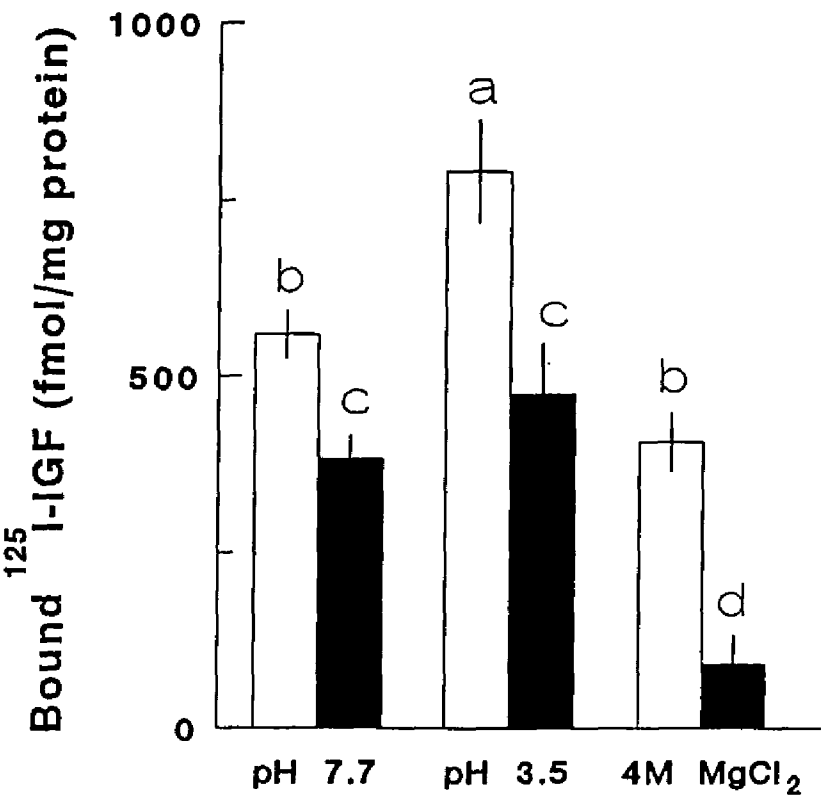

Figure 10a. Total (open bars) and specific (solid bars) binding in membrane preparations preincubated in Tris pH 7.7 or 3.5, or 4M $MgCl_2$ for 10 min before the binding assay. Values are means ±S.E.M. of 3 replicates for each treatment. Means with the same letter (a,b for total and c,d for specific binding) are not significantly different from each other ($p > 0.05$, DMRT).

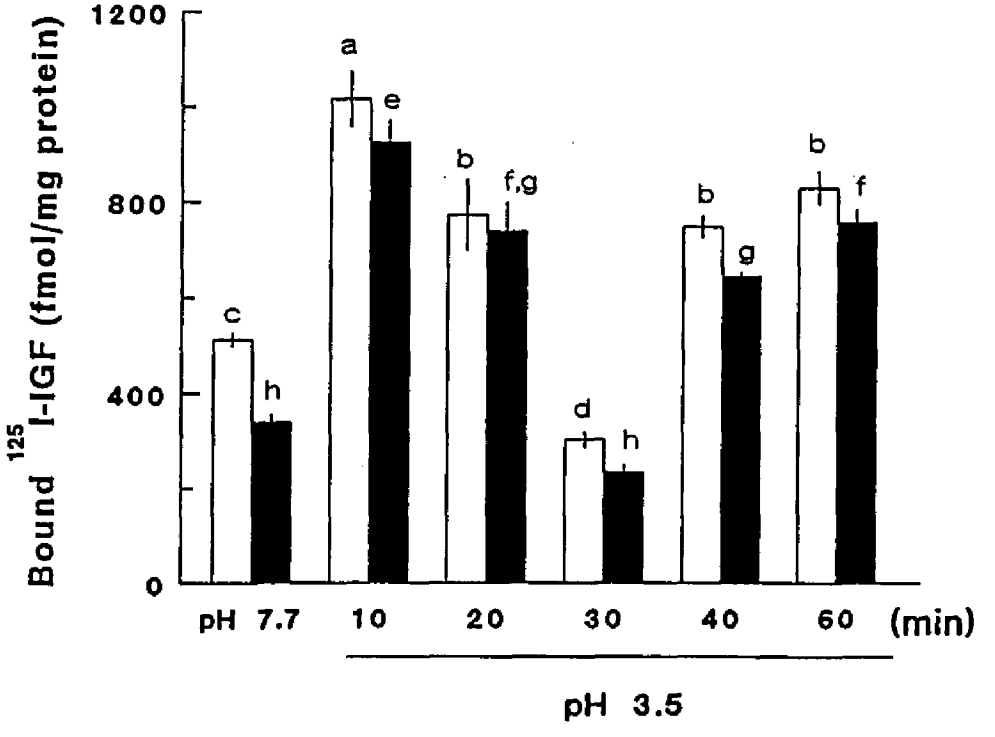

Figure 10b. Total (open bars) and specific (solid bars) binding by membrane preparations which were preincubated in Tris pH 3.5 for different lengths of time before the binding assay. Values are means ±S.E.M. of 3 replicates for each treatment. Means with the same letter (a-d for total and e-h for specific binding) are not significantly different from each other ($p > 0.05$, DMRT).

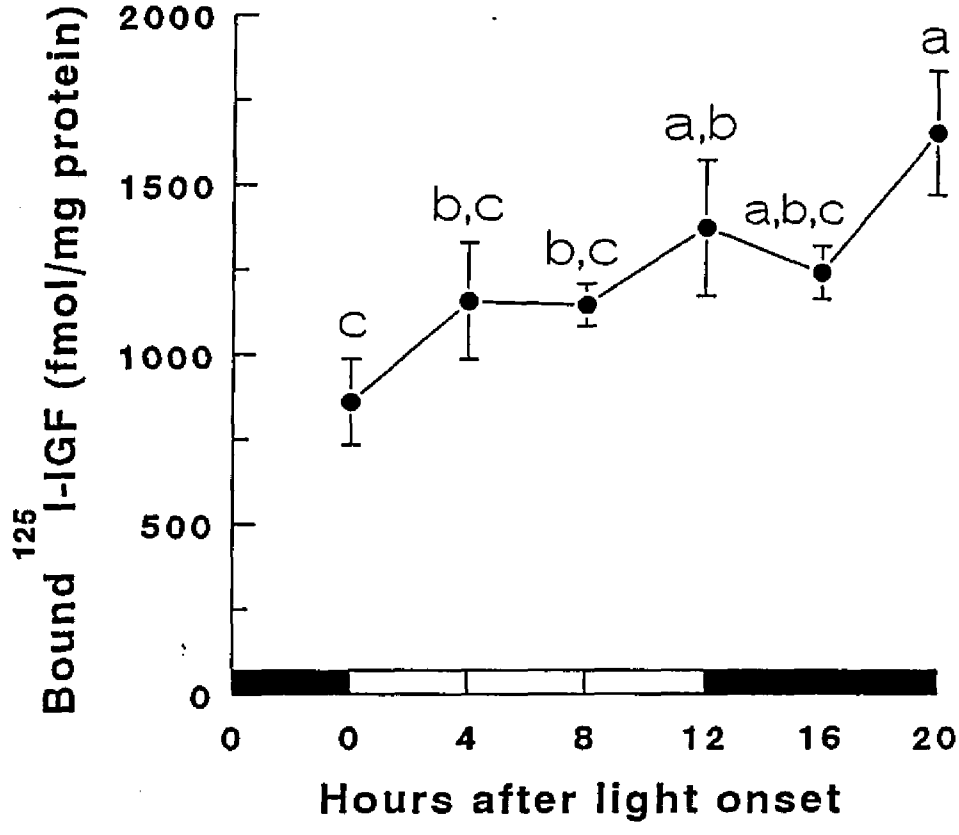

Figure 11. Total binding of ^{125}I-IGF-I in tissue of fish sacrificed at different times during a 24-h period. Values are means ± SEM of five fish with 3 replicates for each fish. Means with the same lower case letters are not significantly different ($p > 0.05$, DMRT). Solid bars indicate period of darkness.

onset (0 h) and the highest binding (1653 ±183 fmol/mg protein) occurred during the late dark period 4 h before light onset (20 h). The binding at light offset and during the dark hours were higher than the daily total mean value of 1239 ±107 fmol/mg protein. Values at light onset and during the light hours were lower than the daily mean value.

Displacement with unlabeled IGF-I and insulin. Binding of ^{125}I-IGF-I is competitively inhibited by increasing the concentrations of unlabeled IGF-I (10^{-12}-10^{-7} M) (Figure 12). On the other hand bovine insulin (10^{-12}-10^{-7} M) has little inhibitory effect on the binding of labeled IGF-I. With bovine insulin about 30% displacement was attained at the highest concentration used (10^{-7} M).

Saturation binding assay. Saturation binding studies were performed at the time points of minimum (light onset) and maximum (4 h before light onset) binding, and at 6 h after the onset of light. Analyses of IGF-I binding yielded curvilinear Scatchard plots (Figure 13a-c) indicating the presence of two binding components: high affinity low capacity and low affinity high capacity. The F tests indicate that the two-site model fit the data better than the one-site model ($F_{2,4} = 69$, $p < 0.001$; $F_{2,5} = 17$, $p < 0.01$ and $F_{2,5} = 15$, $p < 0.01$ for the three time-points respectively). IGF-I binding is known to

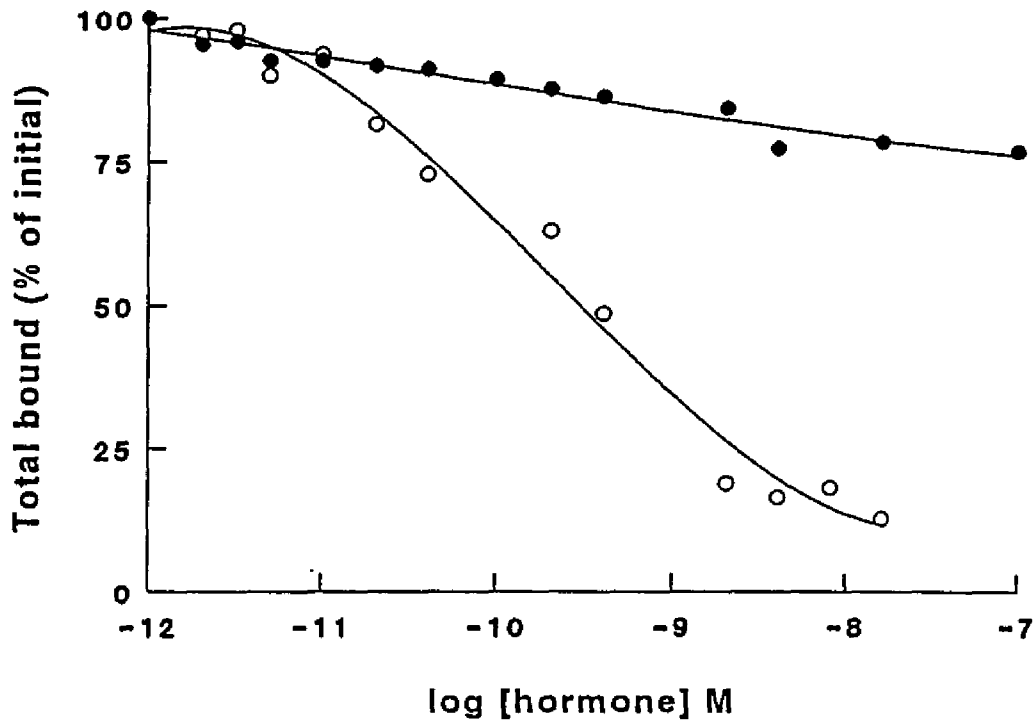

Figure 12. Displacement of ^{125}I-IGF-I by unlabeled IGF-I (O) and bovine insulin (●). Membranes were incubated with ^{125}I-IGFI (0.06 nM) and increasing concentrations of the unlabeled hormones are 10^{-12} - 10^{-7} M.

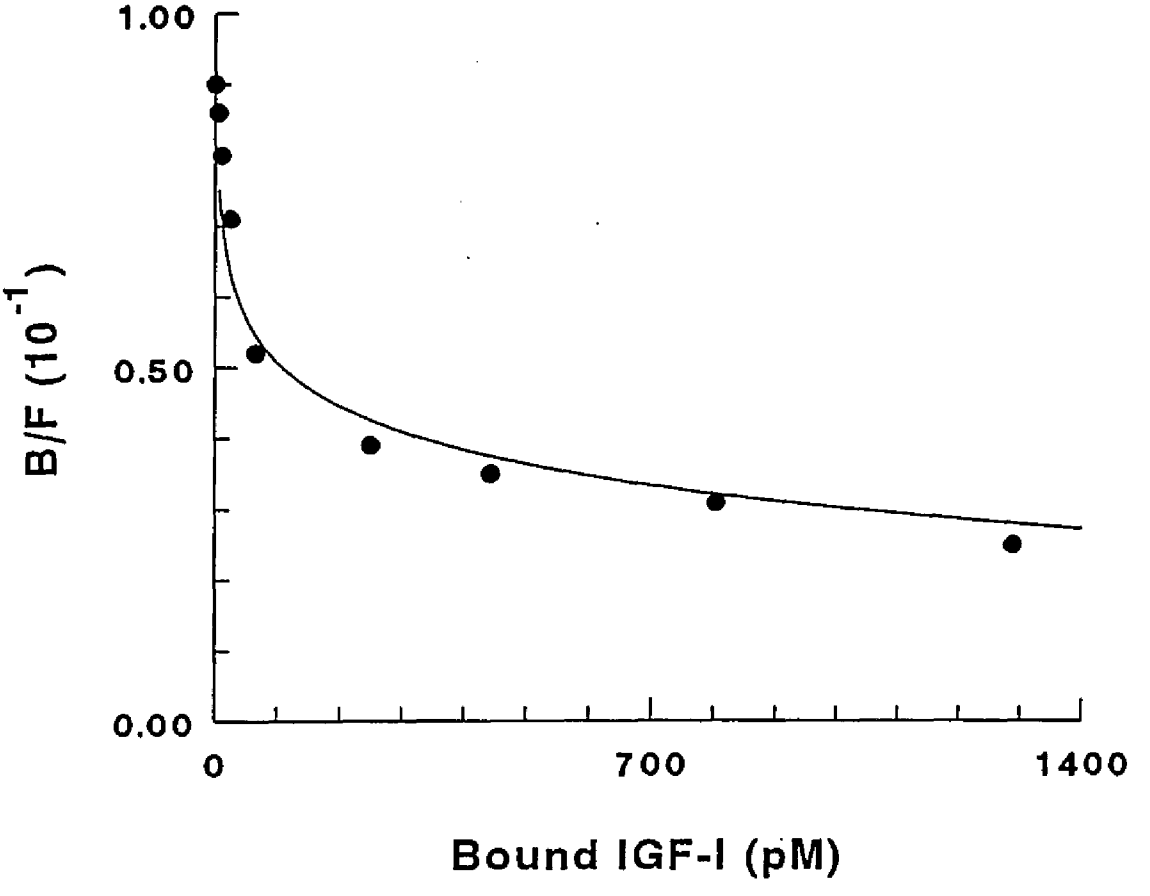

Figure 13a. Scatchard plot of ^{125}I-IGF-I binding to muscle homogenates of fish sacrificed at light onset. Values are means ± SEM of 3 separate determinations. The concentration of ^{125}I-IGF ranged from 0.04 t0 53 nM

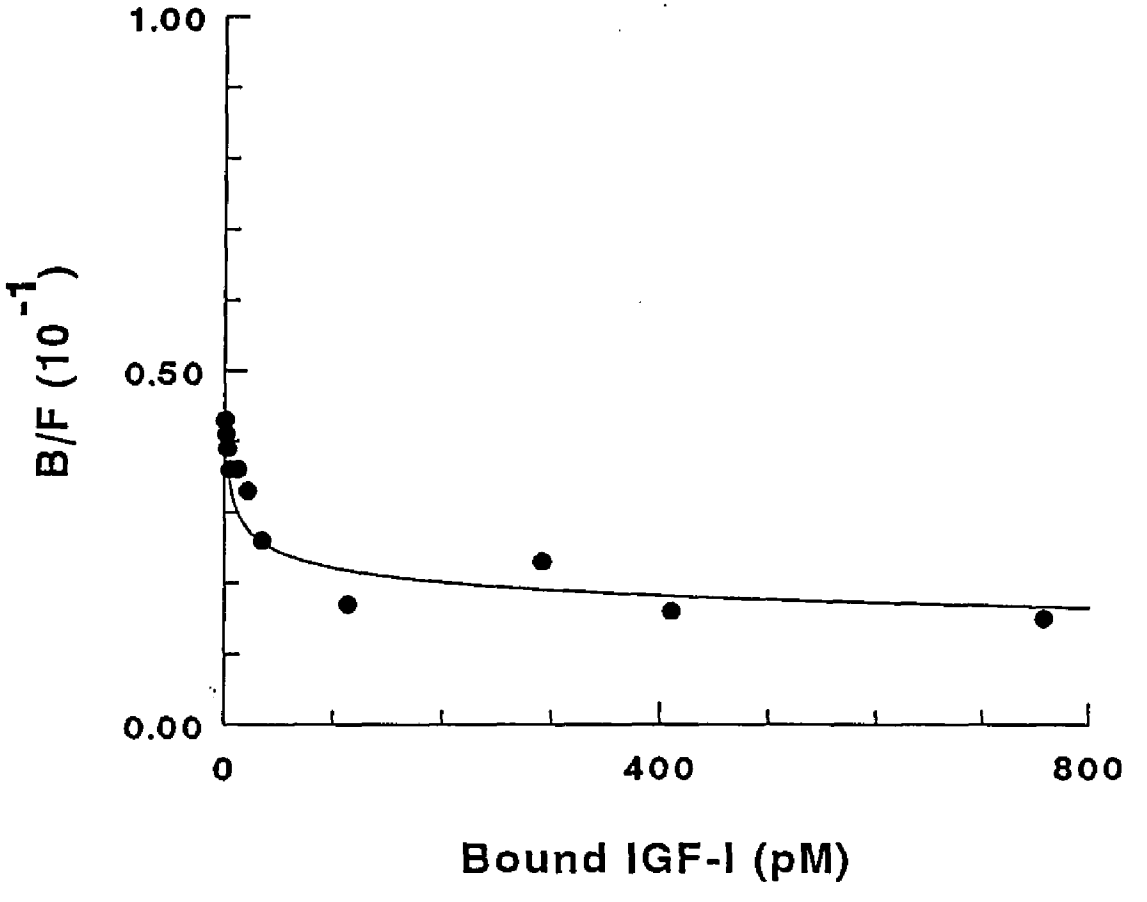

Figure 13b. Scatchard plot of ^{125}I-IGF-I binding to muscle homogenates of fish sacrificed 6 h after light onset. Values are means ± SEM of 3 separate determinations. The concentration of ^{125}I-IGF ranged from 0.04 t0 53 nM

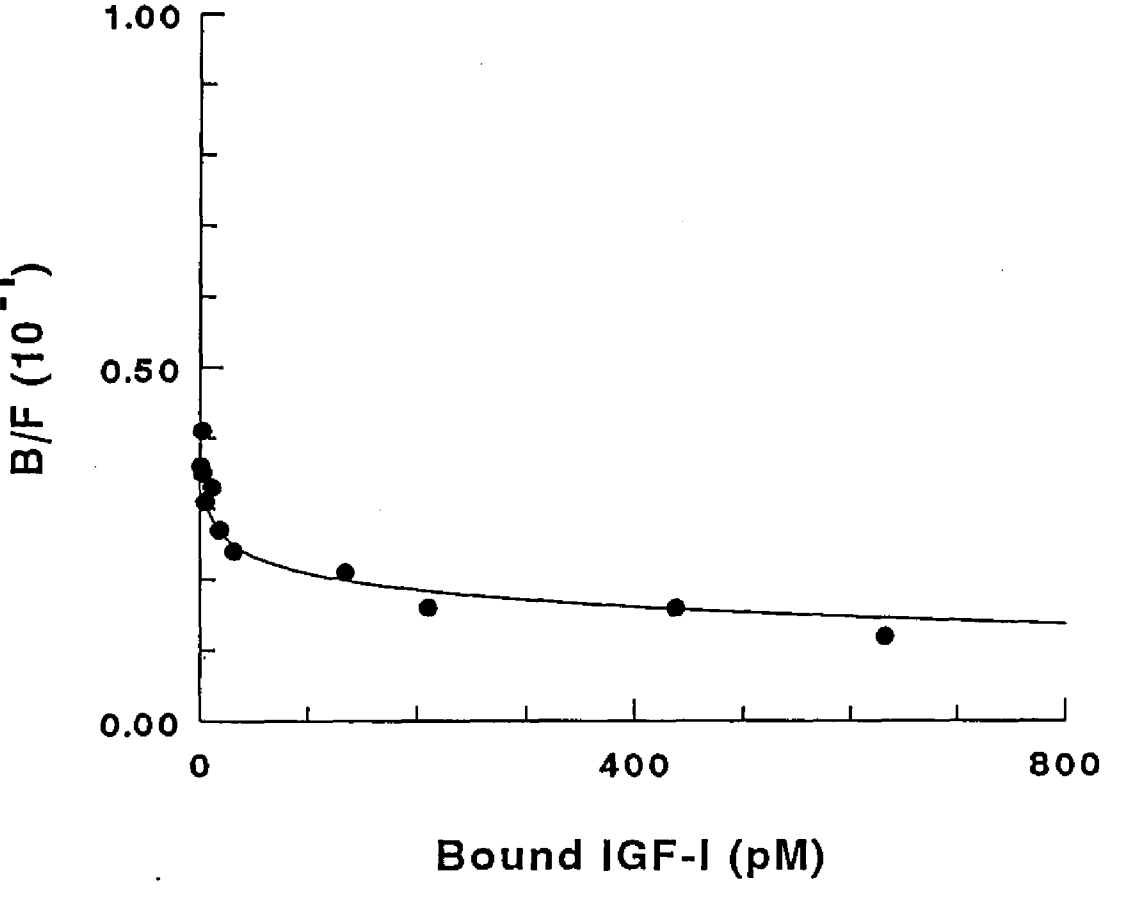

Figure 13c. Scatchard plot of ^{125}I-IGF-I binding to muscle homogenates of fish sacrificed 20 h after light onset. Values are means ± SEM of 3 separate determinations. The concentration of ^{125}I-IGF ranged from 0.04 t0 53 nM

exhibit curvilinear Scatchard plots as a result of negative cooperativity (Christoffersen, *et al.*, 1994).

The binding parameters were estimated by Scatchard analysis of the data for the different time points. The binding affinities (Kd) of the high affinity low capacity component were 3.13 ±1.4, 3.58 ±0.8, and 1.93 ±0.45 nM respectively for the three time points. The Kd at 20 h tends to be relatively lower (that is higher affinity) than at other times of day coinciding with the time of greatest binding observed in Figure 11. Estimates of receptor density/binding sites (Bmax) are 100 ±13 (0 h), 86 ±22 (6 h) and 45 ±18 (20 h) fmol/mg membrane protein. The receptor density shows a decreasing order with the lowest value at 20 h. The apparent difference in receptor density was not statistically significant.

The affinities of the low affinity system were 36.3 ±12.2, 114.8 ±26.7 and 52 ±13.6 nM for the 3 times (0, 6 and 20 h after light onset). The highest Kd (that is lower affinity) was found 6 h after the onset of light. The daily difference of Kd was statistically significant ($p<0.05$). Estimates of receptor density for 0, 6 and 20 h after light onset were 1104 ±392, 1276 ±306 and 706 ±202 fmol/mg membrane protein, respectively.

Discussion

This study demonstrates the presence of binding sites for ^{125}I-IGF-I in membrane preparations of muscle of the gulf killifish. Receptors for IGF-I have been described in a wide range of tissues of animals that have been investigated (e.g. Duclos and Goddard, 1990; McFarland, *et al.* 1992). Information about the presence and tissue distribution of IGF-I receptors in fish is lacking. Recently Drakenberg, *et al.* (1993) have reported IGF-I receptors in brain tissue of the sea scorpion (*Cottus scorpio*) and other lower vertebrates. The result of the present study and that of Drakenberg, *et al.* (1993) indicate that IGF-I receptors may have a wide range of tissue distribution in fish as well.

The binding of ^{125}I-IGF-I in the present study exhibited time dependency when incubated at 20°C with equilibrium reached at about 1 h of incubation. The time of equilibriation for IGF-I reported in the literature show that it is dependent on the incubation temperature. For example in human fibroblasts cultured at 15 or 30°C, equilibrium was attained after 2 h and 30 min respectively (Rosenfeld and Dollar, 1982). In chicken liver membrane preparation equilibrium was attained at 30 min and 14 h at incubation temperatures of 20 and 4°C respectively (Duclos and Goddard, 1990). A time of 1 h to attain equilibrium

observed in this study closely approximates these reported times.

Treatment of membrane preparations with Tris pH 3.5 profoundly increased the binding of ^{125}I-IGF-I in the present study. Pretreatment of membrane preparation with acidified Tris (pH 3.5) has been employed to strip away already bound ligand (Zapf, *et al.* 1994). Acidic treatment is also often used to extract IGF-I from tissue (D'Ercole, *et al.* 1984), and to separate IGF-I from the IGF-I binding proteins in blood (Daughaday, *et al.* 1982; Binoux, *et al.* 1984; Gargosky, *et al.* 1993). Thus the observed increase in binding of labeled IGF-I in membranes treated with Tris pH 3.5 is perhaps a result of stripping away of already bound ligand. This will increase the free or available receptor number for binding.

Pretreatment with 4M $MgCl_2$ on the other hand seems to have negatively affected the binding ability of the IGF-I receptor. 4M $MgCl_2$ has been used successfully to strip off endogenously bound prolactin in membrane fractions of rat liver (Kelly, *et al.*, 1979) and with partial success to strip bound growth hormone in fish liver (Gray and Kelley, 1991). According to Gray and Kelley (1991) the treatment was effective with salmon and tilapia, but not effective in membrane preparations of *Gillichthys mirabilis*. Thus the results of the present study do not support a use of 4M

$MgCl_2$ to strip off IGF-I from cell membranes of *Fundulus grandis*.

Daily variations in responsiveness to stimuli have been reported for various physiological processes in fish. These include: variations in body weight and or fattening (Lee and Meier, 1967; Joseph and Meier, 1971; de Vlaming and Sage, 1972; Weld and Meier, 1983), hepatic ^{14}C-acetate incorporation (Horseman and Meier, 1979a), hepatic RNA synthesis (Horseman and Meier, 1979b), gonad weights (Weld and Meier, 1983 and 1984; Emata, *et al.*, 1985) and immune response (Nevid, 1993) to various timed daily administration of hormonal and nonhormonal stimuli. The studies presented in Chapter 2 show responsiveness of muscle tissue to IGF-I was also variable during a day. Studies in non-teleosts indicate that variations in responsiveness to stimuli may be attributed to daily changes in receptor number and affinity (Cincotta and Meier, 1985; Scheving, *et al.*, 1989).

The binding experiment performed over a 24 h period in the present study demonstrates daily variations of IGF-I binding (Figure 11). The daily variation in IGF-I binding may reflect differences in receptor abundance and/or relative receptor affinities. A daily variation in the circulating level of IGF-I can also affect the number of free receptor sites available for binding by the labeled ligand. This possibility is overcome, or at least

minimized, in the present study by treating membrane preparations with Tris pH 3.5. Thus, the contribution of the circulating level of IGF-I to the observed daily variation in the amount of bound label is thought to be minimal.

Comparison of the daily pattern of IGF-I binding (Figure 11) with the Scatchard analyses of receptor numbers and affinities suggests that daily variations in affinities could be more responsible than variations in receptor densities for daily variations in binding. That is greatest affinities and lowest densities occurred 20 h after light onset when binding was greatest. However, a more definitive assessment of the relative contributions of these receptor characteristics is not justified on the basis on the results of the present study.

Daily variations in receptor number with no significant changes in affinity have been reported for insulin (Cincotta and Meier, 1985), melatonin (Gauer, *et al.* 1992) and serotonin (Hulihan-Giblin, *et al.* 1993) receptors. On the other hand changes in both receptor number and affinity have been demonstrated for epidermal growth factor (Scheving, *et al.* 1989). Daily changes in receptor number or abundance may result from variations in rates of synthesis and degradation, or relative availability of receptors for binding which may result from modulation of membrane properties. For example, it has

been shown that cells whose membranes have been enriched in unsaturated fatty acids (oleate and linoleate) have an increased number of insulin receptors and decreased receptor affinity (Ginsberg, *et al*. 1981).

IGF-I and insulin are known to have distinct high affinity receptors. Each one binds with the highest affinity to its own receptors, and binds poorly to the other's receptors. Several reports show that insulin binds to IGF-I receptor only at high concentrations. It displaces binding of IGF-I at concentrations greater than 10^{-8} M (Beguinot, *et al*. 1985; Blazer-Yost, *et al*. 1989) or 10^{-7} M (Gammeltoft, *et al*. 1985). The result of the present study is in agreement with these reports. The greatest displacement of ^{125}I-IGF-I binding (30%) was found at a concentration of 10^{-7} M, the highest concentration used in this study. Although displacement of insulin by IGF-I was not examined in this study, many reports dealing with mammalian studies provide evidence that IGF-I displaces insulin bound to insulin receptor very poorly. The reports of other investigators and the result of the displacement experiment in the present study indicate that the displacement of insulin and binding of IGF-I to insulin receptors is negligible. The binding of ^{125}I-IGF-I in the present study is believed to be to IGF-I receptors.

The results of the present study show the presence of specific IGF-I binding receptors in muscle of the gulf

killifish, and that the binding of IGF-I to membrane preparations of muscle varied during the day. No definitive conclusions can be drawn about the relative contributions of the daily changes in receptor number or affinity to the daily variation in the binding of ^{125}I-IGF-I. This needs to be investigated further. Highly purified membrane preparations may be useful in resolving whether or not receptor number and/or affinity are determinants of the daily variation in responsiveness of muscle cells to IGF-I.

SUMMARY

The results of this study show that the rate of protein synthesis (amino acid incorporation into protein) varies during the day in the several tissues examined (scale, muscle, liver and intestine). It was also found that the time-of-day of peak protein synthesis may differ with the tissue studied. Scale and muscle displayed the same pattern of label incorporation in male and female fish. On the other hand liver and intestine showed variable patterns in male and female fish. Scale and muscle showed peak incorporations during the scotophase and early photophase respectively. The period of maximal incorporation corresponds well with the reported intervals when hormones involved in protein synthesis (growth hormone, prolactin and IGF-I) show peak circulating levels. Growth hormone and prolactin have peak circulating levels during the scotophase or early photophase (eg. Leatherland, *et al.* 1974, Spieler, *et al.* 1979; Bates, *et al.* 1989). Somatomedin-C (IGF-I) exhibit increased levels at the onset of light (Emata, 1990). The daily patterns of glycine incorporation in scale and muscle may thus reflect the daily rhythms of hormones that are involved in protein synthesis.

In addition to hormone rhythms tissue responsiveness to stimuli such as hormones, also vary during a day. Thus the daily variation in the rate of protein synthesis

observed in the present study may arise as a consequence of daily variations of tissue responsiveness to hormones that affect protein synthesis.

The present study also examined in muscle if the responses of the tissue to treatments with growth hormone, prolactin and IGF-I vary during the day. Growth hormone and prolactin have variable time-of-day dependent effects on glycine uptake by muscle tissue *in vivo*. Growth hormone produced the greatest response when given at the onset of light. Prolactin was also most effective when administered at the onset of light.

IGF-I also showed variable time-of-day dependent effects on glycine uptake by muscle. Treatment of muscle with IGF-I *in vitro* (tissue culture) produced greatest incorporation of the label in tissue that was prepared at light onset. The responses were less when muscle was taken from fish sacrificed at 6 and 12 h after the onset of light. This peak of responsiveness to growth promoting hormones corresponds with the time of peak glycine incorporation for muscle in untreated fish reported in Chapter 1.

The daily variation in incorporation of labeled glycine in untreated fish and the variation in responsiveness of muscle to the different hormones may be attributed to daily changes in tissue sensitivity and/or responsiveness to hormones occurring in the responding

tissue. Changes in receptor number and/or affinity may account for the daily variations in responsiveness of tissues. This was explored by binding studies using membrane preparations of muscle homogenate and ^{125}I-IGF-I.

Binding of ^{125}I-IGF-I exhibited time dependency. When incubation was performed at 20°C equilibrium binding was attained near 1 h of incubation. The binding of the tracer was greatly affected when the tissue homogenates were treated with either Tris-HCl pH 3.5 or 4M $MgCl_2$. Pretreatment of membrane preparations with Tris pH 3.5 significantly enhanced specific binding whereas 4M $MgCl_2$ reduced the total and specific binding. Thus pretreatment of membrane preparations with Tris-HCl pH 3.5 appears to be an effective means of stripping off endogenously bound IGF-I. This may provide a better estimate for determining the total receptor number.

A competitive binding study using bovine insulin as a competitor to the tracer IGF-I shows that insulin competes poorly with the tracer for IGF-I receptors. Although this is not a direct test, it suggests that the tracer IGF-I binds mainly to IGF-I receptors.

A daily variation in the binding of tracer IGF-I to muscle homogenate was found in this study indicating that variations in receptor properties may account for a variation of tissue responsiveness. The binding studies do not provide conclusive evidence as to whether changes in

receptor number or affinity has the major role. The results suggest that changes in affinity may be more determinant.

It is hypothesized that the phase relations between stimulus rhythms and of rhythms of tissue responsiveness to a stimulus determine the nature and/or magnitude of the response to a stimulus. This stimulus/response interaction has been demonstrated to have important regulatory roles for lipid metabolism and reproductive activity in fish and other animals (for review Meier, 1984 and 1992). Collectively, the results of the present study suggest that the phase differences between the rhythms of hormones that are involved in the regulation of protein metabolism and the rhythms of tissue responsiveness to these hormones perhaps have important roles as well in the regulation of growth and development. Such a tissue specific interaction of stimulus and response may account for growth rates of an organism and for differential growth rates of organs during development.

The present findings demonstrate a temporal dimension in endocrine regulation of growth. Growth rates are regulated by an interaction of hormone rhythms and rhythms of responses of tissues to these hormones.

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APPENDIX

Letter of Permission

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VITA

Zeleke Negatu was born in Debre Marcos, Ethiopia. He obtained his B.S. and M.S. degrees in Biology and Zoology, respectively, from Addis Ababa University (AAU) at Addis Ababa, Ethiopia. He joined the Department of Biology (AAU) after obtaining his B.S. degree, and has served as instructor in the Department until he joined Louisiana State University. He was awarded a three year Fellowship from the Louisiana Methodist Scholarship Program that enabled him to join Louisiana State University for a Ph. D. program in the Fall of 1988.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Zeleke Negatu

Major Field: Physiology

Title of Dissertation: Daily Variations in Protein Synthesis and Tissue Responsiveness to Growth Hormone, Prolactin and Insulin-like Growth Factor-I in the Gulf Killifish, Fundulus grandis Baird and Girard

Approved:

Albert H. Meier
Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

John W. Lynn

Thomas Dietz

John T. Caprio

Kevin Kleinow

Date of Examination:

November 4, 1994